Food Research

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THE VIABILITY OF *ESCHERICHIA COLI* AND A SPOILAGE YEAST IN CARBONATED BEVERAGES

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(Manuscript received January 11, 1957)

Soft drink beverages are unique in being among the few packaged foods which do not, in general, receive any type of process as a deterrent to spoilage. Rather, the soft drink industry depends upon the inherent growth inhibiting characteristics of the product and good plant sanitation to prevent spoilage. Any physical or chemical property of a soft drink may potentially have an effect on the viability of microorganisms in that beverage, but to determine the basic pattern of preservative action, factors essentially common to all beverages are of main concern. Sucrose concentration, acidity, and degree of carbonation are the salient factors of most beverages and, parenthetically, the latter two are considered the major characteristics upon which the prevention of microbial spoilage rests.

Commercial experience has led to two rules of thumb which are applied to the survival of microorganisms in carbonated soft drink beverages: (1) spoilage is reduced by increased acidity and carbonation, and (2) the microbial count in a carbonated beverage decreases with increased storage time. In substantiation of these "rules" quantitative information, although highly desirable, is very sparse. Although the separate effects of carbonation, pH, and sugar concentration on the viability of microorganisms have been previously studied, very little information is available on their combined effects or on their effects as related specifically to soft drinks.

Very early, the papers in the field of sanitation and bacteriology showed recognition of the fact that various organisms died faster in carbonated water and beverage than in uncarbonated water *(13, 17, 29)*. In 1924, J. R. Donald, C. L. Jones, and A. R. M. MacLean *(6)* stated that in a beverage carbonated with sufficient pressure, contaminating bacteria would not multiply and gradual sterilization would be accomplished with storage. This work led to the impression that bacteria could not survive at greater than approximately 4.0 volumes of carbonation. Yeast also died when subjected to carbonation *(24, 27)*; hence, the packer of a highly carbonated drink felt relatively secure from spoilage regardless of other conditions. This lethal effect was limited to carbonation pressure and not just pressure per se, for test organisms survived comparable atmospheres of nitrogen *(6)*. Later it was shown that inert gases required thousands of pounds per square inch pressures to produce germicidal effects *(15, 16)*. More recently, N. F. Insalata *(14)* reconfirmed the sterilizing effect of carbonation on both a bacterium and a yeast and showed that the germicidal effect increased with increasing carbonation.

Concentrations of sucrose necessary to produce a germicidal action on microorganisms are well above the range found in soft drink beverages. Gen-

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erally, the germicidal sucrose concentration was set at about 60 to 70% for bacteria and yeasts *(8, 22, 28)* and as high as 80% for sugar tolerant yeasts *(1)*. At sucrose concentrations found in carbonated beverages a protective action against the destruction of microorganisms might even be exerted *(20)*. When both acid and sugar were employed as preservatives the acid was the more important *(8, 9)* and as suggested by Levine and Fellers *(18, 19)* it may be that apart from the indirect effect of altering the H-ion concentration, sugar addition aids but little the toxic effect of an acid on bacteria and yeasts. The effect of acid alone on *Escherichia coli* has been extensively studied *(2, 3, 7, 21)* and Forgacs and Tanner *(12)* showed that apple juice at pH 4.12 containing 140,000 *E. coli* organisms per ml. was sterile in 114 hours and that death was logarithmic. With acetic acid, a pH of approximately 2.5 was required to effect a preservative action against a number of strains of the yeast *Zygosaccharomyces globiformis (1)*. Sensitivity of yeasts and bacteria to pH varies widely according to the specific organism; however, in general, yeasts are more aciduric and less sensitive to changes in pH than are the bacteria.

In this investigation the viabilities of a spoilage yeast and *Escherichia coli* were measured at several combinations of sucrose concentration, pH, and degree of carbonation which encompassed the range found in soft drinks. From these experiments the basic pattern of action of the 3 main beverage variables on microbial lethality was estimated.

EXPERIMENTAL METHODS

Escherichia coli (C. C. C. No. CO-4) and a yeast (C. C. C. No. Y-34)[b] which had been isolated from a spoiled, carbonated orange drink were used as the test organisms. This strain of yeast was selected from a collection of yeasts obtained over a period of time from spoiled soft drinks. It showed desirable growth characteristics on agar media and possessed the ability to grow in the range of sucrose concentration and pH studied.

The experimental work was organized into 2 phases according to the test organism used: phase I *(E. coli)* and phase II (yeast). A total of 48 runs was made in each phase, including 32 different combinations of pH (2, 3, 4, and 4.5), degree Brix (0, 5, 10 and 15), and volumes carbonation (0, 2, 3, and 4.5) of which 8 combinations were run in triplicate.

To simulate commercial canning conditions, and also for convenience, the test variables were packed in soft drink cans using the laboratory canning and bottling equipment. Composition of the syrup "throw" and the pressure under which the carbonated water was added were carefully predetermined to result in a final product of the desired composition. Strict adherence to commercial procedures was maintained throughout: city water was chemically treated, filtered, and titrated to the proper balance; bottlers' sugar (sucrose) was used; the proper volume of 50% citric acid solution prepared from the reagent chemical was incorporated in the syrups to produce a final concentration of 0.02N, the average citrate concentration present in soft drinks. Citric acid was chosen because it is the most commonly used acidulant for soft drinks and is an acid of relatively low toxicity *(25)*. The desired pH values were obtained by adjusting the "throw" with 1N solutions of HCl and NaOH.

Method of canning and inoculating. A portion of the *E. coli* (phase I) runs was packed in 12-ounce flat top cans. Following the addition of syrup the cans were filled, inoculated, and closed. Inoculum was an 18-hour lactose broth culture appropriately diluted with M/40 phosphate buffer (pH 7.0) to result in a final cell concentration of 10,000 organisms/ml. in the finished product. Inoculum dilutions were based on direct counts made in a Petroff-Hausser chamber using a high dry dark phase contrast objective. Phosphate buffer was mandatory for all *E. coli* work because preliminary tests showed

[b] The authors regret that the yeast used in this investigation was inadvertently lost before its characterization and identity was fully determined. Unfortunately, the culture is no longer available for future studies.

that unbuffered laboratory distilled water was highly toxic to the organisms. After closure, cans were thoroughly shaken and incubated at room temperature. At appropriate time intervals, one can from each run was opened, 1 ml. was plated on Difco Tryptone-Glucose extract agar, and the plates were incubated 24 hours at 98° F. before counting.

A second portion of the *E. coli* (phase I) runs was packed in 6-ounce Cap Sealed cans. Variables were packed and closed without inoculation, and all cans, except the 4.5 vol. CO_2 runs, were pasteurized at 140° F. for 30 minutes. Cans were than cooled to 35° F. in a controlled temperature refrigerator, caps removed, inoculum added, and finally re-capped with sterile crowns as quickly as possible.

The 6-ounce Cap-Sealed can method was used for all yeast experiments. Inoculum consisted of a 3-day orange serum broth culture diluted with distilled water to yield 10,000 cells/ml. in the finished product. At appropriate times, samples were plated out using Orange Serum agar (O.S. agar No. 77-Exchange Orange Products Company) and counted after 2 days' incubation at 86° F.

Each variable was checked for pH, Brix and carbonation as part of the rigid schedule of controls maintained throughout the investigation.

Method of calculating results. Recorded data for each variable were plotted with the logarithm of the number of viable organisms per ml. (X) as the ordinate and the time of incubation (t) as the abscissa. Microorganisms under the influence of adverse agents die according to the mathematics of a first order reaction *(23)*; hence, a semilog death-time plot should yield a straight line. Therefore, a best straight line was drawn through the data points using the "Method of Averages" *(4)*. A differential equation of this line in terms of the two variables, number of organisms (X) and time (t), is

$$\frac{d^2 \log X}{dt^2} = O \qquad (1)$$

which upon integration gives an expression for the rate of change of one variable with respect to the other.

$$\frac{d \log X}{dt} = -k \qquad (2)$$

In this expression k is the constant of integration and is called the death rate constant. Integration of this differential expression gives the equation of the line, which rearranged and solved for k gives

$$k = \frac{\log Xo - \log X}{t} \qquad (3)$$

where k = death rate constant
X = number of organisms/ml. at time t
Xo = number of organisms/ml. at zero time

The death rate constant is a reaction rate constant where the reaction is the death of an organism. High values of k indicated a high lethality and k was equal to zero when the experimental conditions were not unfavorable to the survival of the organism being tested. The value of k was expressed in reciprocal units of time.

Using equation (3) the death rate constant was determined for each experimental run. For clarification the completed treatment of the data for *E. coli* run No. 7 is given in Figure 1.

To determine the time necessary for a given per cent reduction of the initial contamination, equation (3) was solved for t with log X_o − log X set equal to 1 for a 90% reduction of the original number of organisms, 2 for a 99% reduction, etc.

Sample estimates of the population standard deviation for the controls were obtained by taking the square root of the sample estimate of variance, using n-1 degrees of freedom *(26)*.

RESULTS

E. coli (phase I). Death rate constants (k) for *E. coli* under variable conditions of pH, degree Brix, and volumes of carbonation are given in Table 1.

Although *E. coli* is a relatively unimportant organism from the standpoint of the spoilage of a carbonated beverage, it is of major importance from a public health standard standpoint. With this in view the times necessary to reduce a given initial contamination

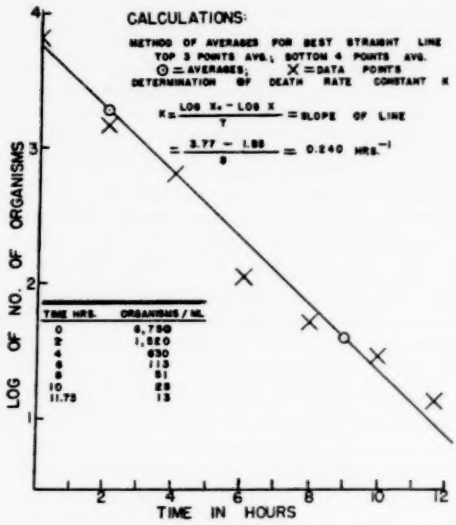

Figure 1. Treatment of data for E. coli, run no. 7 (pH = 3; ° Brix = 10; vol. carbonation = 0).

level of *E. coli* to conform with the U. S. Public Health Service standards for drinking water were calculated, using the death rate constants in Table 1. These values are given in Table 2.

A cursory glance at the data of either Table 1 or Table 2 reveals that pH was the major factor in eliminating *E. coli*. Comparing averages of the death rate constants, the lethality at pH 2.0 was approximately 50 times that at pH 3.0, about 250 times that at pH 4.0, and roughly 900 times that at pH 4.5.

At pH 2.0 the presence of sucrose exerted a slight protective action against the pH effects, since an increase in degree Brix produced a decrease in lethality. At pH 3.0, 4.0, and 4.5, 12 comparisons of the effect of increasing the degree Brix at a constant carbonation were possible. Of these, 5 showed a decrease, 6 showed an increase, and one showed no change with an increase in degree Brix. Any change in a variable necessitated comparing 2 separate runs, each subject to normal experimental variations. Lethality was considered independent of a given variable when a change in the variable resulted in a change in lethality being equally likely in either direction. Therefore, lethality was considered independent of the sucrose concentration at a pH of 3.0 or greater.

Increasing carbonation at pH 4.5 and any given sucrose concentration decreases the time necessary to kill *E. coli* (Table 2), whereas at lower pH values the death rate was independent of the carbonation. At pH 4.5 and various constant sucrose concentrations 14 comparisons of the effect of increased carbonation on lethality can be made. In 13 of these the lethality increased with increasing carbonation; in one no change in lethality was observed with changing carbonation. In the 3 lower pH values tested of the possible 18 comparisons of varying carbonation, 11 showed a decrease in lethality and 7 showed an increase in lethality with increased carbonation. This distribution was consistent with the estimation that at these pH values the lethality was independent of the volumes of carbonation. It was possible that at the lower pH values, which were dramatically more toxic, the effect of carbonation was masked, whereas, at a pH of 4.5, which was not markedly toxic, the effect of carbonation became important. In all uncarbonated variables at pH 4.5 the organisms grew, but only one carbonated variable at this pH survived.

Summarily, pH was the major factor affecting the survival of *Escherichia coli* in a simulated carbonated beverage. The effect of sucrose modified the toxicity of pH only at low pH; the toxic effect of carbonation was apparent only at high pH.

TABLE 1

The effect of various conditions of degree Brix, pH, and volume of carbonation on the viability of E. coli

Run number	pH	Variables ° Brix	Vols. CO_2	Death rate const. (k) in hrs.$^{-1}$ x 10^2
1, 17, 33	2.0	0	0	870
37	2.0	0	2	666
2, 18, 41	2.0	0	3	805
45	2.0	0	4.5	859
21	2.0	5.0	0	546
22	2.0	5.0	3	333
3, 25, 35	2.0	10.0	0	294
39	2.0	10.0	2	600
4, 26, 43	2.0	10.0	3	275
47	2.0	10.0	4.5	353
29	2.0	15.0	0	375
30	2.0	15.0	3	261
5	3.0	0	0	8.35
6	3.0	0	3	6.96
7	3.0	10.0	0	24.0
8	3.0	10.0	3	6.96
9	4.0	0	0	0.655
10	4.0	0	3	1.04
11	4.0	10.0	0	3.62
12	4.0	10.0	3	1.74
13, 19, 34	4.5	0	0	0
38	4.5	0	2	0.672
14, 20, 42	4.5	0	3	1.29
46	4.5	0	4.5	2.45
23	4.5	5.0	0	0
24	4.5	5.0	3	0.605
15, 27, 36	4.5	10.0	0	0
40	4.5	10.0	2	0
16, 28, 44	4.5	10.0	3	0.66
48	4.5	10.0	4.5	0.80
31	4.5	15.0	0	0
32	4.5	15.0	3	0.80

Yeast (phase II). Values of the death rate constants in experiments where yeasts were the test organism are given in Table 3. Table 3 was arranged in a slightly different order from Table 1 to emphasize the importance of carbonation.

The importance of carbonation in preventing yeast spoilage of a carbonated beverage was immediately apparent from the data in Table 3. Without carbonation there was no lethality of the yeasts regardless of the degree Brix or pH. In all 20 of the carbonated variables except one, a lethal effect was shown.

Rigorous examination of the data did not reveal any consistent modifying effect of any kind in the lethality of yeasts with variation of degree Brix. In the uncarbonated variables the pH was not lethal at any level tested, but in the carbonated variables the pH significantly affected the lethality. At constant degree Brix and volumes carbonation, a comparison of yeast lethalities at varying pH showed that in 16 out of a total of 18 possible comparisons, the greater the pH the less was the lethality. An increase of one pH unit resulted in an average decrease in the death rate constant of 0.08/hour.

Controls. Variables of the experiments, although assigned a discrete theoretical value, exhibited a degree of variation. By analysis of a series of controls these variations were determined and recorded in Table 4 for the experiments employing *E. coli* and in Table 5 for experiments employing yeast.

TABLE 2

The hours required to reduce an initial contamination of 100 E. coli/ml. to meet the U. S. Public Health Service standards for drinking water under various conditions of pH, ° Brix, and volumes carbonation

Volumes carbonation	° Brix	hours			
		pH = 2.0	pH = 3.0	pH = 4.0	pH = 4.5
0	0	0.46	47.0	610	.....
	5	0.73	.....	.....	.....
	10	1.36	16.7	110	.....
	15	1.07	.....	.....	.....
2	0	0.60	.....	.....	595
	10	0.67	.....	.....	.....
3	0	0.50	57.5	385	310
	5	1.20	.....	.....	660
	10	1.45	57.5	230	606
	15	1.53	.....	.....	500
4.5	0	0.47	.....	.....	163
	10	1.13	.....	.....	500

DISCUSSION

Death rate constants for *E. coli* varied from 0 to 8.70/hour over the range of variables tested while the death rate constants for the test yeast varied from 0 to only 0.01/hour. Hence, under their respective most lethal conditions *E. coli* died at 870 times the rate of the yeast. A comparison of the data Table 1 and 3 also shows that the *E. coli* were more sensitive to changes in the tested variable than were the yeasts.

Based on results of this investigation, soft drink packers would not experience growth in the beverage, of the two spoilage organisms tested, if that beverage had a pH of 4.0 or less and was carbonated at 2.0 or more volumes. Most soft drinks, with the exception of some citrus flavors, meet these requirements for non-growth.

Most lethal condition for yeasts was in solution with a pH of 2.0, 10° Brix and 4.5 volumes of carbonation. If a beverage of essentially this composition (no beverage we know of is so acid and few beverages have so high a carbonation) was infected with 100 yeasts per ml. of the strain tested in this investigation, 17 days would be required to reduce this contamination to one yeast per 100 ml. Under less lethal conditions (pH of 4.5, 3 volumes carbonation, and 10° Brix) 119 days would be required to affect this 99.9% reduction of the contaminating yeast. Therefore, for almost 4 months there would be a substantial number of yeasts present, all of which would presumably be metabolizing and producing gas and other resultant end products. The product would be essentially sterile after this storage time and very likely it would also be spoiled due to the accumulation of off-flavor producing end products and excessive carbonation. Therefore, statements in prior literature to the effect that carbonation will produce sterility, although essentially true, could not with the same reliability be rephrased to say that carbonation will prevent spoilage.

Insalata *(14)* in his paper on this subject concluded, with a very limited

TABLE 3

The effect of various conditions of degree Brix, pH, and volumes of carbonation on the viability of yeast

Run number	Vols. CO_2	Variables pH	° Brix	Death rate const. (k) in hrs.$^{-1}$ x 10^2
1, 17, 33	0	2.0	0	0
21	0	2.0	5	0
3, 25, 35	0	2.0	10	0
29	0	2.0	15	0
5	0	3.0	0	0
7	0	3.0	10	0
9	0	4.0	0	0
11	0	4.0	10	0
13, 19, 34	0	4.5	0	0
23	0	4.5	5	0
15, 27, 36	0	4.5	10	0
31	0	4.5	15	0
37	2	2.0	0	0.25
39	2	2.0	10	0.45
38	2	4.5	0	0.30
40	2	4.5	10	0
2, 18, 41	3		0	0.37
22	3	2.0	5	0.35
4, 26, 43	3	2.0	10	0.51
30	3	2.0	15	0.35
6	3	3.0	0	0.27
8	3	3.0	10	0.25
10	3	4.0	0	0.21
12	3	4.0	10	0.16
14, 20, 42	3	4.5	0	0.24
24	3	4.5	5	0.20
16, 28, 44	3	4.5	10	0.14
32	3	4.5	15	0.15
45	4.5	2.0	0	0.77
47	4.5	2.0	10	1.00
46	4.5	4.5	0	0.16
48	4.5	4.5	10	0.22

number of experiments, that sterility was accomplished in fewer days where the Brix was lower, and theorized that this may be attributed to the fact that the lower sugar content (8.5% instead of 12%) afforded less nutrient. The optimum sugar content for the growth of both yeast and bacteria is approximately 1 to 2% *(10, 11)*. This investigation did not confirm the variation of lethality with Brix as found by Insalata.

Results of this investigation are limited in the strict sense to the particular strains of *E. coli* and yeast used as the test organisms. Bottles and cans have been known to fail under the extreme carbon dioxide pressures produced by a contaminating yeast in the beverage and some bacteria have been grown in extremely acid environments. However, from a practical standpoint, the results represent a fairly accurate picture of what might be expected in different carbonated beverages depending upon their degree Brix, pH, and carbonation.

TABLE 4

Controls on the variables used in experiments determining the viability of E. coli

Variable	Assigned value	Number of samples	Mean	Standard deviation	95% Confidence limits
pH	2.0	34	2.0	0.032	2.0 ± 0.06
	3.0	4	3.0	0	3.0
	4.0	4	4.0	0	4.0
	4.5	31	4.58	0.096	4.58 ± 0.19
Vols. of CO_2	0	No Controls Measured			
	2	11	2.1	0.24	2.1 ± 0.48
	3	32	3.2	0.19	3.2 ± 0.38
	4.5	10	4.42	0.197	4.42 ± 0.39
° Brix	0	No Controls Measured			
	5	4	5.0	0	5.0
	10	13	10.0	0	10.0
	15	4	15.0	0	15.0

TABLE 5

Controls on the variables used in experiments determining the viability of yeast

Variable	Assigned value	Number of samples	Mean	Standard deviation	95% Confidence limits
pH	2.0	64	2.05	0.05	2.05 ± 0.10
	3.0	15	3.0	0	3.0
	4.0	18	4.05	0.09	4.05 ± 0.18
	4.5	65	4.55	0.06	4.55 ± 0.12
Vols. of CO_2	0	No Controls Measured			
	2	11	2.1	0.24	2.1 ± 0.48
	3	32	3.2	0.19	3.2 ± 0.38
	4.5	10	4.42	0.197	4.42 ± 0.39
° Brix	0	No Controls Measured			
	5	4	5.0	0	5.0
	10	29	9.96	0.17	9.96 ± 0.34
	15	4	15.2	0.5	15.2 ± 1.0

Any given carbonated beverage contains other factors that may influence the death rate of a potential spoilage organism. Substances which may function as inhibitors in a carbonated beverage in addition to those studied are: ethers and esters which naturally occur in fruit juices; different essential oils and spices *(5)*; peel oil in citrus drinks; extracts from roots and herbs in root beer, birch beer, and sarsaparilla; foaming agents; capsicum in some ginger ales; alcohol used in extracting flavors; glycerine used to dissolve flavors; emulsifying agents; coloring agents; and the type of acid used (other than citric acid) in acidifying *(21, 25)*. Any of these factors may alter the basic lethalities given in this paper. However, it should be emphasized that no combination of beverage factors can serve as a replacement for good plant sanitation in preventing spoilage.

SUMMARY

Lethalities of the bacterium *E. coli* and of a yeast were measured under 32 different combinations of variables; pH (ranging from 2.0 to 4.5), ° Brix

(ranging from 0 to 15), and carbonation (ranging from 0 to 4.5 volumes). *E. coli* was markedly more sensitive to changes in the variables and more susceptible to the toxicity of the variables than was the test strain of yeast.

The pH variable exerted the major effect on the survival of *E. coli*. Lethality at pH 2.0 was approximately 50 times that at pH 3.0, 250 times that at pH 4.0, and 900 times that at pH 4.5. At a pH greater than 2.0, lethality was independent of sucrose concentration, whereas at pH 2.0 increased sucrose appeared to exert a slight protective effect. Increased lethality of *E. coli* was experienced with increased carbonation at pH 4.5, but the lethality was not significantly dependent upon carbonation at a pH less than 4.5.

Survival of yeasts in the simulated soft drink beverage was primarily a function of the carbonation. Without carbonation there was no lethality regardless of the ° Brix or pH. Lethality of the yeast was independent of the sucrose concentration. In the carbonation variables an increase in pH caused a significant decrease in lethality.

The significance and limitations of these findings in relationship to the packaging of soft drink beverages is discussed.

LITERATURE CITED

1. Bell, T. A., and Etchells, J. L. Sugar and acid tolerance of spoilage yeasts from sweet cucumber pickles. *Food Technol.*, **6**, 468–471 (1952).
2. Cohen, B. The effect of temperature and hydrogen-ion concentration on the viability of *B. coli* and *B. typhosum* in water. *J. Bacteriol.*, **7**, 183–230 (1922).
3. Dagley, S., Dawes, E. A., and Foster, S. M. Influence of pH value and aeration on the growth of *Aerobacter aerogenes* and *Bacterium coli* in defined media. *J. Gen. Microbiol.*, **8**, 314–322 (1953).
4. Davis, D. S. *Empirical Equations and Nomography*. 1943. McGraw-Hill Book Co., New York, N. Y.
5. Dold, H., and Knapp, A. The antibacterial action of spices. *Z. Hyg. Infektionskrankh.*, **128**, 696–706 (1948).
6. Donald, J. R., Jones, C. L., and MacLean, A. R. M. The effect of carbonation on bacteria in beverages. *Am. J. Public Health*, **14**, 122–128 (1924).
7. Douglas, M., and Edin, M. D. Some principles regulating the death of pathogenic intestinal bacteria in artificial media and in fruit juices. *Lancet*, **219**, 789–791 (1930).
8. Erickson, F. J., and Fabian, F. W. Preserving and germicidal action of various sugars and organic acids on yeasts and bacteria. *Food Research*, **7**, 68–79 (1942).
9. Fabian, F. W., and Wadsworth, C. W. Experimental work on lactic acid in preserving pickles and pickle products. II. Preserving value of acetic and lactic acids in the presence of sucrose. *Food Research*, **4**, 511–519 (1939).
10. Fink, H., and Lechner, R. Production of food yeast in wood-sugar solutions. III. The natural nitrogen content of wood-sugar wort. *Biochem. Z.*, **286**, 83–90 (1936).
11. Fink, H., Lechner, R., and Heinisch, E. Production of food yeast in wood-sugar solutions. II. *Biochem. Z.*, **283**, 71–82 (1935).
12. Forgacs, J., and Tanner, F. W. Longevity of pathogenic bacteria in apple juice. *Fruit Prod. J.*, **22**, 295–299 (1943).
13. Gershenfeld, L. Bacteria in (so-called) soft drinks. *Am. Food J.*, **15**, 16–17 (1920).
14. Insalata, N. F. CO_2 versus beverage bacteria. *Food Eng.*, **24**, 84 (1952).
15. Johnson, F. H., and Lewin, I. The disinfection of *E. coli* in relation to temperature, hydrostatic pressure, and quinine. *J. Cellular Comp. Physiol.*, **28**, 23–45 (1946).
16. Johnson, F. H., and Lewin, I. The influence of pressure, temperature, and quinine on the rates of growth and disinfection of *E. coli* in log growth phase. *J. Cellular Comp. Physiol.*, **28**, 77–97 (1946).
17. Koser, S. A., and Skinner, W. W. Viability of colon-typhoid group in carbonated water and carbonated beverages. *J. Bacteriol.*, **7**, 111–121 (1922).

18. LEVINE, A. S., AND FELLERS, C. R. Action of acetic acid on food spoilage and microorganisms. *J. Bacteriol.*, **39**, 499–513 (1940).
19. LEVINE, A. S., AND FELLERS, C. R. Inhibiting effect of acetic acid on microorganisms in the presence of sodium chloride and sucrose. *J. Bacteriol.*, **40**, 255–269 (1940).
20. MCFARLANE, V. H. Behavior of microorganisms in fruit juices and in fruit juice—sucrose solutions stored at −17.8° C. (0° F.). *Food Research*, **7**, 509–518 (1942).
21. MORRISON, G. A. Anaerobic growth of *E. coli* in the presence of certain acids. *Nature*, **171**, 937–938 (1953).
22. NUNHEIMER, T. D., AND FABIAN, F. W. Influence of organic acids, sugars, and sodium chloride upon strains of food poisoning Staphylococci. *Am. J. Public Health*, **30**, 1040–1049 (1940).
23. RAHN, O. The problem of the logarithmic order of death in bacteria. *Biodynamica*, **4**, 41–130 (1943).
24. SCHMITTHENNER, F. Action of carbon dioxide of yeasts and bacteria. *Weinbau. Wiss. Beih.*, **3**, 147–187 (1949).
25. SHILLINGLAW, C. A., AND LEVINE, M. Effect of acids and sugars on the viability of *E. coli* and *Eberthella typhosa*. *Food Research*, **8**, 464–476 (1943).
26. STEARMAN, R. L. Statistical concepts in microbiology. *Bacteriol. Rev.*, **19**, 160–215 (1955).
27. SWEARINGEN, J. S., AND LEWIS, I. M. The nature of the effect of CO_2 under pressure on bacteria. *J. Bacteriol.*, **26**, 201–210 (1933).
28. TARKOW, L., FELLERS, C. R., AND LEVINE, A. S. Relative inhibition of microorganisms by glucose and sucrose syrup. *J. Bacteriol.*, **44**, 367–372 (1942).
29. WIPPLE, G. C., AND MAYER, A. On the relation between oxygen in water and longevity of the typhoid bacillus. *J. Infect. Dis.*, Supplement No. 2, 282–401 (1906).

ANALYSIS OF THE PROTEIN CONSTITUENTS OF DRIP FROM THAWED FISH MUSCLE

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Fishery products preserved by freezing potentially have characteristics more like those of fresh fish than do products preserved by any other process currently in use. Despite the widespread acceptance of frozen fishery products, however, a decided consumer preference for fresh fish often exists. The reasons for this preference may be attributed to a progressive deterioration in quality of the frozen product, particularly after prolonged cold storage. When fish muscle is frozen and subsequently thawed, a cloudy, liquid phase called *drip* exudes from the muscle. In addition, when the thawed muscle is cooked, the surface of the cooked flesh often is coated with an unsightly white deposit of coagulated drip proteins, commonly referred to as *curd*. Further, the thawed fish when cooked often toughens and becomes fibrous as compared with cooked fish that has never been frozen. The rate of quality deterioration in frozen fish is related not only to cold storage conditions but also to species differences.

Several different theories have been proposed concerning the origin of drip in frozen fish muscle and reviews dealing with this problem and factors influencing the production of drip have been discussed elsewhere *(3, 7)*. The most recent evidence *(7, 14, 17)*, however, would suggest that drip is due to denaturation of proteins which normally tend to hold the water of the muscle and to changes in the cellular structure of the muscle brought about by the formation of ice. The general effect of freezing and the rate of freezing on the fine structure of fish muscle has been demonstrated by histological techniques, although experimental findings often appear rather contradictory. Love *(10)* recently has suggested that the appearance of desoxypentosenucleic acid (DNA) in the expressible fluid from thawed muscle indicates rupture of the cell membrane, with liberation of nuclear material.

The fact that disintegrated fresh tissue does not liberate an appreciable quantity of muscle juice, however, would suggest that the mere rupture of the cell membrane in itself does not fully explain the release of large quantities of drip when the same material is frozen and thawed. It has been demonstrated *(14)* that the retention of cellular fluids or drip is related to the power of muscle proteins to imbibe free liquid. This imbibing power is influenced by electrolyte content and by pH and a zone of minimum effect is exhibited in the regions of the isoelectric points of the fish muscle proteins *(17)*. The aggregation or loss of solubility of actomyosin—the principal contractile protein of the myofibril—by freezing, and its subsequent inability to rehydrate has been demonstrated by many workers *(6)*. Dyer *(6)* reports that the sarcoplasmic or non-contractile proteins, however, show no change of solubility on freezing except after very long storage. The denaturation of the contractile proteins was also suggested by Mahadevan and Carter *(12)*, who detected a permanent and constant decrease in volume of fish flesh after freezing and thawing. Salt dehydration of fish muscle, wtih accompanying shrinkage of

tissue fibers *(1)* and denaturation of actomyosin *(6)*, also has been demonstrated.

These findings suggest that a disturbance of the intracellular hydrated contractile protein equilibrium rather than that of the sarcoplasmic fraction is at least in part responsible for the liberation of drip from thawed fish muscle. It is felt that a better knowledge of the protein constituents of drip could offer supporting evidence to this theory. Thus, if it could be concluded that the sarcoplasmic protein fraction is that fraction which constitutes drip and shows no change in its ionic characteristics during freezing, the origin of drip could be more definitely attributed to contractile protein denaturation.

The objectives of this study were to determine the protein constituents of drip comparative to the known major protein fractions of fish muscle and to detect any relative changes in the ionic characteristics of these proteins when fish muscle is frozen. The procedures employed were conventional fractionation and electrophoretic methods to determine the types of proteins present in drip and to compare the electrophoretic mobilities of drip proteins and the same proteins isolated from fresh fish muscle.

EXPERIMENTAL

Material and methods. *Preparation of drip and extracts.* All of the experiments reported in this paper were carried out on drip or extracts isolated from the white muscular tissue of yellow-striped rockfish *(Sebastodes nebulosus)*. The preparation of drip was standardized according to the following procedure: Seven rockfish were caught and held at 0° to 4° C. for 24 hours, after which time the fish had passed through *rigor*. The fish were filleted and skinned, the small amount of lateral red tissue removed, and the fillets passed once through a food grinder. The coarsely ground flesh was thoroughly mixed and distributed in capped, glass centrifuge tubes. The samples of ground flesh were frozen at −20° C. and held at this temperature for approximately 60 days before thawing. As needed, the samples were thawed by placing the centrifuge tubes under cold running tap water for 30 minutes. The samples were centrifuged for 30 minutes at 4000 R.C.F. at 0° C. The supernatant fluid was filtered by gravity through a medium porosity sintered glass funnel. This centrifuge drip contained approximately 4% protein by biuret *(16)* analysis and was used directly for qualitative fractionation tests and for electrophoretic analysis.

In order to obtain only the sarcoplasmic fraction, tissue extractions at low ionic strength of fresh fish muscle for the comparative tests were carried out essentially according to the method described by Dingle, Eagles, and Neelin *(5)*. These extracts were obtained by adding unbuffered 0.1 M. KCl to an equal weight of coarsely ground fresh, post-*rigor* rockfish muscle and allowing the mixture to stand unstirred overnight at 0° to 2° C. The extracts were centrifuged and filtered as described for the preparation of centrifuge drip. These extracts contained approximately 2% protein and were used directly for electrophoretic analysis.

Paper-electrophoretic analysis. Paper-electrophoretic analysis was carried out in a horizontal, open, strip-type apparatus (Biophysical Instruments Inc.). Whatman 3MM chromatography paper cut into 12.5 x 300 mm. strips proved the most satisfactory of various papers tested. The procedure was briefly as follows: Four paper strips (2 duplicates) were soaked for 30 minutes in the electrophoresis electrolyte solution (phosphate buffer, ionic strength 0.05 at a selected pH of 5.50, 6.50, or 7.50). The papers were blotted briefly and carefully pulled taut on the removable paper rack. A 2 mm. glass rod was rolled under the paper strips to form an apex or tent 100 mm. from the cathode end of the paper. The rack was placed in the electrophoresis cabinet, and the glass cover (a sheet of Whatman 3MM paper was glued to the under side of the cabinet lid to prevent condensation from forming and dripping onto the paper strips) replaced. The power was turned on and the system allowed to equilibrate for 30 minutes. The cover was removed and the appropriate amount (0.010 ml. of a 2% solution) of protein solution was applied at the apex of the paper by means of a Spinco sample striper. The cover was replaced

again, and the experiment continued for 17 hours. In all but preliminary experiments, an applied potential of 100 or 150 volts, giving a current of 3–5 milliamperes, was used. The electrophoretic analysis was carried out in a household-type refrigerator maintained at approximately 0° C. After completion of a run, the rack containing the paper strips was placed in an oven held at 120° C. for 30 minutes. Following the procedure described by Durrum *(2)*, the papers were stained in the bromophenol blue-zinc sulfate-acetic acid mixture, washed in 2% acetic acid, dried, and scanned at 575 mμ in a Beckman model B spectrophotometer equipped with a Beckman 8300 model B chromatogram attachment.

RESULTS

Preliminary qualitative tests. Rupture of the cell membrane and disorganization of the intracellular myofibrils by freezing would be expected to liberate cytoplasmic constituents. Differential centrifugation up to 25,000 R.C.F. of centrifuge drip [made up to 0.88M. *(9)* sucrose] for particulate cell components, however, did not reveal the presence of any large cytoplasmic granules (mitochondria). Homogenization of intact, thawed, white rockfish muscle with 0.88 M. sucrose, followed by differential centrifugation, did yield an extremely small yellowish pellet at 25,000 R.C.F., microscopically appearing quite granular, unlike rat liver mitochondria *(9)*. Love *(11)* reports a trace of similar appearing material centrifuging out of the expressible fluid of cod fish at 24,000 R.C.F. and containing about 6% of the total DNA phosphorus. He concludes that expressible fluid is free from large particles (mitochondria) but contains DNA-protein aggregates of nuclear origin. Thus, if mitochondria are present in the cellular structure of the white muscle of fish, they are present in such small quantity that their presence in centrifuge drip would seem questionable, particularly due to their unstable characteristics.

The possible presence of the contractile protein actomyosin of the myofibril in drip was also determined. Centrifuge drip was diluted with 9 volumes of ice-cold water, or, alternatively, was dialyzed against frequent changes of buffer solution of pH 7.3 and ionic strength of 0.05. The absence of any precipitating protein in either of these tests definitely indicates *(8)* the absence of actomyosin in drip.

Removal of electrolytes by dialysis of the centrifuge drip against cold distilled water, however, resulted in a considerable amount of precipitated protein. When the protein precipitate was removed, the clear, dialyzed solution gave a large additional quantity of protein precipitate when trichloroacetic acid was added. The protein precipitated by dialysis against distilled water partially redissolved in dilute salt soluton, although a portion, apparently denatured, did not. These tests thus indicated the presence in drip of a globulin fraction, insoluble in water and of low stability, and a water soluble albumin fraction, which is relatively stable. Connell *(4)* reports, however, that such a clear-cut distinction is not possible, in that several of the components of an extract of low ionic strength suffer only a partial loss of solubility on dialysis against distilled water.

These preliminary tests therefore suggested a definite similarity between the protein composition of drip and extracts of low ionic strength from fish muscle *(8)*.

Electrophoretic analysis. Nikkilä and Linko *(13)* obtained satisfactory resolution of proteins and reported finding 6 to 8 components in extracts of low ionic strength from each of different species of fish by paper-electrophoresis at ionic strength 0.05 and pH 7.55 at high voltage (296 v.) in a run of 17 hours. In the present work, however, it was found that gross overlapping, often to the point of complete obliteration of minor bands, occurred when high voltage (300 v.) was used over a period of 17 hours. In addition, the correspondingly higher amperage resulted in local heating and excessive evaporation and condensation. When, however, lower voltages (100 v., for example) were employed for a run of 17 hours, at a pH closer to the isoelectric points of the proteins, the net result was a greater decrease of the mobilities of the slower-migrating proteins than of the faster-migrating ones. In addition, the total migration of the fastest component was essentially the same at high or low voltage, indicating that a position of equilibrium *(2)* was being approached at either voltage for the fastest component. It also was noted that the lowering of the pH from 7.50 to 6.50 not only retarded the migration of the slower-mobility components but caused the appearance of two faster-moving minor components. Thus, Figure 1, illustrating electrophoretic patterns obtained in preliminary studies on centrifuge drip, shows that at pH 7.50 when the voltage was decreased from 300 (Figure 1-A) to 100 volts (Figure 1-B), for example, the separation of the slower mobility components improved. When the pH was lowered to 6.50 and the voltage decreased from

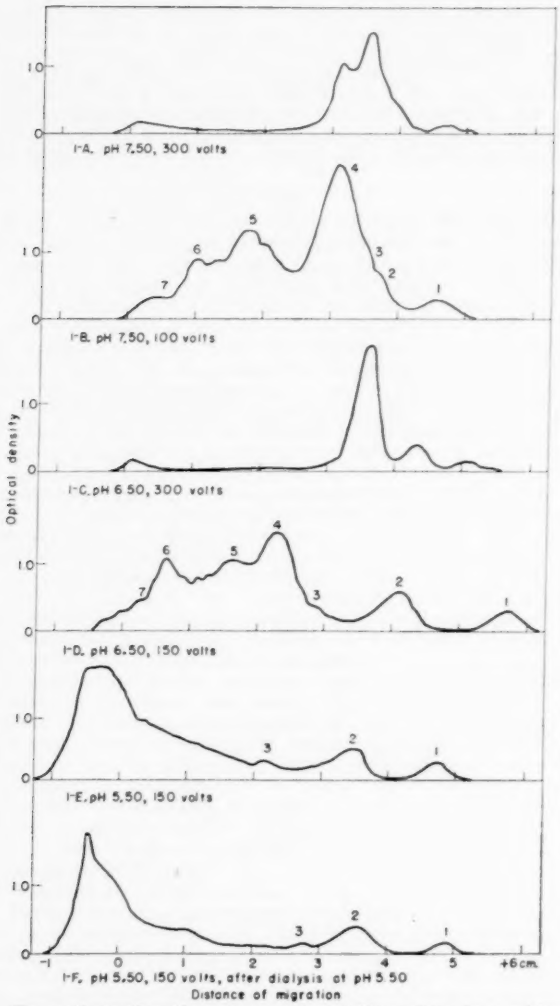

Figure 1. Paper-electrophoretic patterns of centrifuge drip from white muscle of yellow-striped rockfish; phosphate buffer u = 0.05, pH 7.50, 6.50, and 5.50 at high and low voltages, respectively. Duration of electrophoresis, 17 hours.

300 (Figure 1-C) to 150 volts (Figure 1-D), a similar effect was noted. The two new fast-moving peaks are also observed when the pH is thus lowered. The peaks are numbered from 1 to 7 in order of decreasing mobility in Figures 1-B and 1-D for convenience in reference. Peak number 3 was verified by electrophoresis at pH 5.50 (Figure 1-E), where the slower mobility components show little or no migration towards the anode. At this pH (5.50), slight cathodic migration was observed, although the precipitation of protein on the paper obscured any separation of these components. A repeat of this experiment at pH 5.50 after dialysis of the protein solution at pH 5.50 (to remove the

proteins precipitating at this pH) did not indicate any cathodic separation (Figure 1-F). Electrophoresis of drip proteins also was attempted at pH 8.00. At this pH, all of the proteins showed quite similar mobilities at low voltage, thus this higher pH range was not explored further.

Simultaneous electrophoretic analysis of centrifuge drip and the extracts of low ionic strength from fresh rockfish was next carried out for comparative purposes. Typical patterns are recorded in Figures 2-A and 2-B for determinations carried out at pH 6.50 at 150 volts and in Figures 2-C and 2-D for determinations carried out at pH 7.50 at 150 volts. The peaks are numbered from 1 to 7 in order of decreasing mobility. Here again, the patterns obtained by electrophoresis of centrifuge drip at pH 6.50 were more informative than were those carried out at pH 7.50. Thus, 7 components were established in the centrifuge drip obtained from yellow-striped rockfish. Two additional components appearing as minor peaks between peaks 5 and 6 on electrophoresis at pH 6.50 are suspected. Under the condition thus far employed, however, these suspected peaks have been too ill-defined to warrant separate designation. It was noted that only between peaks 4 and 5 did better separation occur at pH 7.50, and at this pH the suspected peaks between components 5 and 6 were no longer apparent. In addition, a gradual tailing off of the slowest part of the patterns often suggested at pH 6.50 the presence of small components slower than component 7.

A comparison of the patterns obtained for centrifuge drip from thawed rockfish and extracts of low ionic strength from fresh rockfish (Figure 2) revealed no qualitative differences as to the number of components present. A quantitative estimation of the

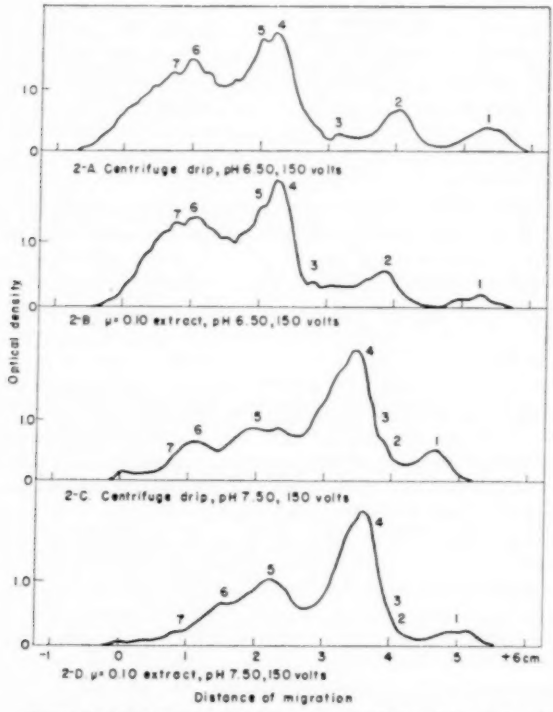
2-A Centrifuge drip, pH 6.50, 150 volts
2-B μ=0.10 extract, pH 6.50, 150 volts
2-C Centrifuge drip, pH 7.50, 150 volts
2-D μ=0.10 extract, pH 7.50, 150 volts
Optical density
Distance of migration
+6cm

Figure 2. Paper-electrophoretic patterns of centrifuge drip and extracts of low ionic strength from the white muscle of yellow-striped rockfish, pH 6.50 and 7.50, at low voltage. Duration of electrophoresis, 17 hours.

protein components was not carried out due to the different dye-binding characteristics of the various proteins. No obvious differences were observed, however, between the relative amounts of each of the comparable components in drip or extract. In addition, insofar as an interpretation of paper-electrophoretic patterns permits, no relative change in the ionic characteristics or mobilities of the component proteins of drip was observed in fish muscle in the frozen state. Thus, when simultaneous electrophoretic analysis of centrifuge drip and extracts of low ionic strength was carried out, the dyed strips were indistinguishable. In repetitive determinations, it was observed that the precision obtained was better for the faster components.

DISCUSSION

Electrophoretic patterns of centrifuge drip and extracts of low ionic strength from rockfish recorded here show marked similarities with patterns recorded by other workers both by paper-electrophoresis *(13)* and by free electrophoresis in solution *(4, 5)* of extracts of low ionic strength from other species of fish. Thus, Connell *(4)* reports the presence of at least 7 components in extracts of low ionic strength from codling muscle which may be divided into Jacob's groups I, II, and III. Group III, of highest mobility, normally contains one small, highly mobile component (peak No. 1). Group II, of average mobility, is divided into 3 components (peak Nos. 2, 3, and 4). Group I is composed of 3 components and usually contributes over half of the total area under the curve (peak Nos. 5, 6, and 7). Peak number 4, reported here, would appear to contribute relatively more to the total area under the curve than Connell *(4)* finds using free electrophoresis. Nikkilä and Linko *(13)*, however, employing paper-electrophoresis for an examination of extracts of low ionic strength from a variety of species of fish, also report a major component in the II group of all species examined. Due to the different staining characteristics of proteins, however, it is difficult to compare the two methods quantitatively.

Dingle, Eagles, and Neelin *(5)* also report a better separation of the proteins present in extracts of low ionic strength of fish muscle when the pH of the electrolyte is decreased. Thus, at pH 5.8, two peaks in group I (corresponding to peaks referred to here as 5 and 6), which apparently represented single components at higher pH values, each separated into two or three distinct peaks. It would appear that group I represents a very complex mixture of proteins.

Recent evidence *(7, 14, 17)* suggests that drip is due in part to denaturation of proteins which normally tend to hold the water of the muscle. In view of the similarity between the number and ionic characteristics of the protein constituents of drip from thawed fish muscle and extracts of low ionic strength of fresh fish muscle and the absence of the contractile protein actomyosin in drip, it is suggested that the sarcoplasmic protein fraction of fish muscle is not intimately associated with the origin of drip. In addition, since it is unlikely that sarcoplasmic proteins are bound to the actomyosin proteins in the intact myofibril, it appears that the contraction of fish muscle on freezing and thawing, with resulting drip formation and texture change, may be at least partially attributable to actomyosin denaturation by a dehydration process. Although it has been shown *(15)* that the freezing process itself produces some subtle change in the structure of actomyosin, yielding a more symmetrical molecule without measurably affecting its solubility, continued frozen storage appears to be responsible for some yet unknown change which causes progressive and irreversible aggregation of the actomyosin.

SUMMARY

The protein constituents of centrifuge drip isolated from thawed fish muscle were determined and compared with the known major protein fractions of fish muscle. A definite similarity between the protein composition of drip and extracts of low ionic strength from fresh fish muscle was noted. Seven components, which may be divided into three mobility groups by paper-electrophoresis, were established in centrifuge drip obtained from the white muscle of yellow-striped rockfish. A comparison of the patterns obtained for centrifuge drip from thawed rockfish and extracts of low ionic strength from fresh rockfish revealed no differences as to the number of components present. No relative change in the mobilities of the component proteins of drip was observed when fish muscle was frozen. In view of the similarity between the number and ionic characteristics of the protein constituents of drip and extracts of low ionic strength of fish muscle, and the absence of the contractile protein actomyosin in drip, it is suggested that the sarcoplasmic protein fraction of fish muscle is not intimately associated with the origin of drip.

LITERATURE CITED

1. Bendall, J. R., and Marsh, B. B. The biochemistry of muscular tissue in relation to drip during freezing. Eighth Int. Cong. Ref., London, 1951. *Mod. Ref.*, **54**, 238 (1951).
2. Block, R. J., Durrum, E. L., and Zweig, G. A manual of paper chromatography and paper-electrophoresis, Part II. 1955. Academic Press Inc., New York, N. Y.
3. Callow, E. H. Frozen meat. *J. Sci. Food Agric.*, **3**, 145 (1952).
4. Connell, J. J. Studies on the proteins of fish skeletal muscle (I). *Biochem. J.*, **54**, 119 (1953).
5. Dingle, J. R., Eagles, D. E., and Neelin, J. M. Electrophoretic studies of proteins extractable from post-*rigor* cod at low-ionic strength. *J. Fish. Res. Bd. Can.*, **12**, 75 (1955).
6. Dyer, W. J. Main problems of fish protein denaturation. *Proceedings*, Symposium on Cured and Frozen Fish Technology, Swedish Inst. for Food Pres. Res., Göteborg, VII, (1953).
7. Empey, W. A., and Howard, A. Drip formation in meat and fish. *Food Preser. Quart.*, **14**, 33 (1954).
8. Hamoir, G. Fish proteins. *Adv. Protein Chem.*, **10**, 227 (1955), Academic Press Inc., New York, N. Y.
9. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E. Cytochemical studies of mammalian tissues (I). *J. Biol. Chem.*, **172**, 619 (1948).
10. Love, R. M. The expressible fluid of fish fillets (I). *J. Sci. Food Agric.*, **6**, 30 (1955).
11. Love, R. M. The expressible fluid of fish fillets (III). *J. Sci. Food Agric.*, **6**, 287 (1955).
12. Mahadevan, V., and Carter, N. M. The complete freezing of fish flesh (II). *Prog. Rep.*, Pac. Coast Station, Fish Res. Bd. Can., (76) 85 (1948).
13. Nikkilä, O. E., and Linko, R. R. Paper-electrophoretic analysis of protein extracted at low-ionic strength from fish skeletal muscle. *Biochem. J.*, **60**, 242 (1955).
14. Seagran, H. L. Reduction of curd in canned salmon prepared from frozen fish (II). *Com. Fish. Rev.*, **18**, (1), 1 (1956).
15. Seagran, H. L. Chemical changes in fish actomyosin during freezing and storage. *Food Research*, **21**, 505 (1956).
16. Snow, J. M. Proteins in fish muscle (II). *J. Fish. Res. Bd. Can.*, **7**, 594 (1950).
17. Tarr, H. L. A. Effect of pH and NaCl on swelling and drip in fish muscle. *J. Fish. Res. Bd. Can.*, **5**, 411 (1942).

ERRORS OF THE SECOND KIND IN AN ACID THRESHOLD TEST

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Difference testing procedures should be based on a consideration of errors of the second kind as well as on errors of the first kind. An error of the first kind is made when it is said that a difference exists when in fact there is no difference. An error of the second kind is made when it is said that no difference exists when in fact there is a difference. Errors of the first kind have been discussed in detail *(3, 4, 5, 6, 10, 11, 12)*. The mathematical model for organoleptic difference testing is the binomial distribution. Errors of the second kind for the binomial distribution are comprehensively treated in the literature *(1, 2, 8, 9)*.

In particular in *(2)* it was shown that, on the basis of errors of the second kind, in threshold tests with sugar solutions it is much more effective to use a few tasters of high acuity than a heterogeneous panel with widely varying abilities of detecting differences.

The purpose of the present study is to examine the effects of errors of the second kind on the design of good detection procedures for determining acid taste thresholds.

ACID TASTE TESTING EXPERIMENT

This experiment was conducted in the spring of 1957 with tartaric acid in water. There were 24 tasters who were students and staff members of the University. Tasting was done each day, usually between 11 A.M. and 12 M. The tasters were told that acid was the taste substance being employed. A dilute, above-threshold, solution was presented to the tasters at the first session for familiarization.

Samples were presented in 125 ml. beakers containing 30 ml. of solution. Five pairs were given at each session. The taster was required to state which solution was the more acid. The order of presentation and the number of low and high concentrations were randomized. Two types of tests were conducted: (a) distilled water versus acid, and (b) acid at one concentration versus acid at ½ the concentration. The concentrations used are given in Table 1. The tartaric acid was C.P. grade.

In the water-acid series, the taster started at concentration 1 and continued to lower concentrations if all the first 15 pairs were correctly identified or to higher concentrations if one or more pairs were incorrectly identified. Unless 4 or more pairs were incorrectly identified at the initial concentration, the test was also continued to lower concentrations. The results of these trials are given in Table 2.

TABLE 1

Concentrations of tartaric acid solutions in water

Number of acid solution	Concentration of tartaric acid in moles per liter
1/32	0.00000293
1/16	0.00000586
1/8	0.00001172
1/4	0.00002344
1/2	0.00004688
1	0.00009375
2	0.00018750
3	0.00037500
4	0.00075000
5	0.00150000

In the acid versus acid tests the initial pair was 2 versus 1. If the 15 pairs at this concentration were correctly identified then pairs one-half as acid were employed. If there were errors in the first 15 pairs presented then pairs of twice the concentration were considered. Unless 4 or more pairs were incorrectly identified at the initial concentration, the tests were also continued to lower concentrations. The test was discontinued when the taster failed to identify 12 or more of the 15 pairs. The data for these trials are presented in Table 3.

ANALYSIS AND DISCUSSION OF THE DATA

An examination of the data in Table 2 suggests that two groups of fairly homogeneous tasters can be considered. The first group is composed of 7 tasters and the second of 17. It is realized within these groups that differences in thresholds and rates of change of detecting abilities exist and that these may fluctuate with time. However, these differences within groups are rather small as compared to the gross differences between groups. Also, in order to point out the importance of considering errors of the second kind it is sufficient to consider these major groupings.

If we consider the rows for totals in Table 2, we see that these averages can be fairly well represented by straight lines for certain ranges of the concentrations. The ranges and lines are given in Table 3. The situation is pictured graphically in Figure 1. It is noted that the x- or concentration-scale

TABLE 2

Paired difference tests on ten tartaric acid solutions for 24 tasters—percent of right choices out of 15 trials

Group	Taster	1/32	1/16	1/8	1/4	1/2	1	2	3	4	5
I	15	—	53	80	100	100	100				
	24	—	—	40	100	100	100				
	2	—	47	64	80	100	100				
	4	40	80	80	93	100	100				
	7	—	60	93	93	100	100				
	6	—	—	62	73	93	100				
	13	—	67	80	100	87	100				
Total (7)		—	58	71	91	97	100				
II	1	—	—	60	87	100	80	100	*	*	*
	3	—	—	58**	67	93	80	100	*	*	*
	5	—	—	—	—	60	60	100	*	*	*
	8	—	40	40	80	80	87	100	*	*	*
	9	—	—	—	—	40	73	100	*	*	*
	12	—	—	—	58**	67	93	100	*	*	*
	14	—	—	58**	67	87	67	100	*	*	*
	20	—	—	—	—	40	80	100	*	*	*
	10	—	—	60	80	100	87	80	100	*	*
	11	—	—	—	—	53	80	80	100	*	*
	16	—	—	—	—	58**	67	87	100	*	*
	18	—	—	—	—	—	47	80	100	*	*
	19	—	—	—	—	—	53	80	100	*	*
	22	—	—	—	60	80	80	73	100	*	*
	23	—	—	—	—	60	67	93	100	*	*
	21	—	—	58**	67	87	93	93	*	*	*
	17	—	—	—	—	—	58**	67	67	73	100
Total (17)		—	—	52	60	68	74	90	98	98	100
Total (24)		—	52	58	69	76	81	93	99	99	100

— Assumed to be 50.
* Assumed to be 100.
** Interpolated.

TABLE 3

Regression lines fit to percent right choices for Groups I and II and both Groups combined (total). The range of validity is indicated by the values of x included

Number of concentration	x	y_I	y_{II}	y_T
1/16	0	58		
1/8	1	71	52	58
1/4	2	91	60	69
1/2	3	97	68	76
1	4		74	81
2	5		90	93
3	6		98	99

$$y_I' = 58.70 + 13.70\,x$$
$$y_{II}' = 41.09 + 9.31\,x$$
$$y_T' = 51.12 + 8.06\,x$$

is logarithmic. It is expected that there is a non-linear effect at either end of the ranges for each of the groupings employed.

If we consider the power, which is 1 minus the probability of an error of the second kind, of the groups I and II and I and II combined for a particular number of trials, 10, and a fixed probability of error of the first kind, 0.05, we get the result pictured in Figure 2. We note, for example, that for concentration 1/4 that the power (probability of detecting a difference) for group I is 0.85, for group II is 0.16 and for the combined groups is 0.31. That is, group I is tremendously more effective in detecting the presence of acid than is group II or the whole panel. We emphasize that the probability of an error of the first kind is constant for all the groups.

We come now to a consideration of the data in Table 4. If our scale for measuring concentrations is correct, we would expect that if a taster could detect a difference with a given probability between 3 and 2 he could also

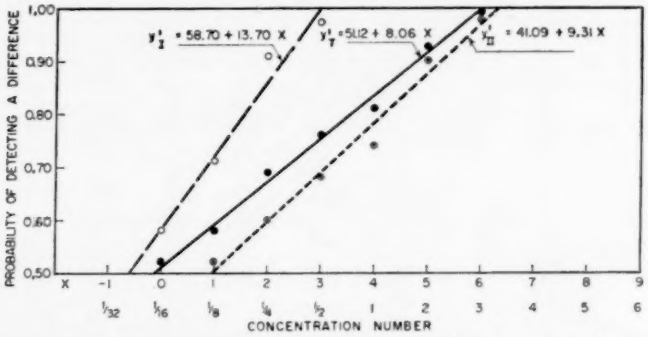

Figure 1. Linear relationships between probability of detecting a difference and concentration of tartaric acid on a log scale for two groups of tasters and the combination of the groups.

Note: A test of the significance between Group I and Group II can be made by comparing the constant terms in the regression lines. The spread of the dots about the regression lines appears to be about the same in both cases. If we assume they are except for random fluctuations we can compute $t = (58.70 - 41.09)/2.10 = 8.39$ with 6 degrees of freedom. Hence, $P < 0.001$.

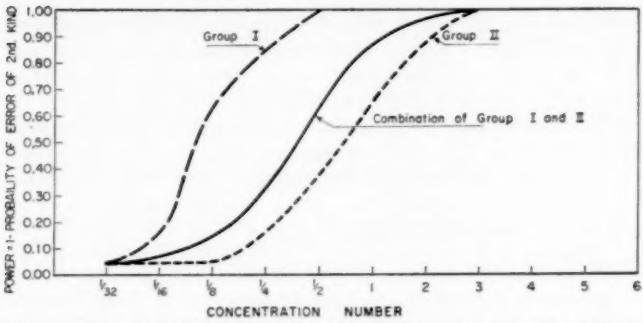

Figure 2. Power of detection procedures for two groups of tasters and the combination of the groups, n = 10, α = 0.05.

detect a difference between 2 and 1 with the same probability. This situation would continue unchanged until the concentrations became so small that they play the role of distilled water; that is, the concentrations are one or both below the taster's threshold.

The data in Table 4 are, of course, subject to random fluctuations but it

TABLE 4

Percent right choices out of 15 trials for one acid solution against the next lower solution for 24 tasters listed in the same order as in Table 1

		Solution comparisons							
Group	Taster	1/16-1/8	1/8-1/4	1/4-1/2	1/2-1	1-2	2-3	3-4	4-5
I	15	67	100	87	100	93	100	—	—
	24	73	100	93	100	100	—	—	—
	2	—	60	80	80	67	93	—	—
	4	73	93	100	100	100	—	—	—
	7	—	93	73	100	100	—	—	—
	6	—	53	93	80	87	100	—	—
	13	—	67	87	93	100	—	—	—
II	1	—	60	100	93	93	100	—	—
	3	—	73	80	93	73	100	—	—
	5	—	—	53	80	93	93	—	—
	8	—	73	93	87	93	100	—	—
	9	—	—	47	73	100	100	—	—
	12	—	53	80	87	87	93	—	—
	14	—	—	67	80	73	100	—	—
	20	—	—	—	67	60	93	—	—
	10	—	—	73	80	73	100	—	—
	11	—	—	—	53	60	93	*	—
	16	—	—	—	—	60	80	100	—
	18	—	—	53	80	80	100	*	—
	19	—	—	—	67	60	100	*	—
	22	—	—	60	80	87	93	*	—
	23	—	60	80	100	80	100	*	—
	21	—	53	80	87	100	*	*	—
	17					60	53	73	87

* Assumed to be 100.

—— Underlining indicates the first comparison after a drop in per cent is expected on the basis of Table 1.

appears that many of the tasters behave as expected. The data on thresholds in Table 2 should be considered in this connection in estimating the points at which ability to detect differences between concentrations can be expected to disappear.

However, there is some evidence that differences at higher concentrations are slightly more detectable for some tasters. To the extent to which this is true, it is indicated that the logarithmic scale is not linear for these tasters beyond the threshold concentrations. More data are needed to explore this acid versus acid situation more fully.

SUMMARY

An organoleptic experiment on the detection of acid in distilled water and the differences between such acid solutions has been presented. From the standpoint of detecting organoleptic differences if they exist, it is important to use the best tasters available.

The acid versus acid part of the experiment gives a check on the linearity of response of the tasters on the commonly used logarithmic scale. It seems that for many tasters the response is approximately linear over a considerable range. However, there is some evidence that for other tasters the response is not linear in that differences at higher levels are more likely to be detected.

We note that the data reported here consist of two independent sets for the same tasters. Typically there are 75 taste trials for each taster presented in Table 2. Independently, there are usually 75 more taste trials presented for the same tasters in Table 4. The results, as far as the two groupings are concerned, are very consistent.

Acknowledgments

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LITERATURE CITED

1. Anderson, R. L., and Bancroft, T. A. *Statistical Theory in Research.* 1952. McGraw-Hill Book Co., New York, N. Y.
2. Baker, G. A., Amerine, M. A., and Roessler, E. B. Errors of the second kind in organoleptic difference testing. *Food Research*, 19, 206–210 (1954).
3. Boggs, M. M., and Hanson, H. L. Analysis of foods by sensory difference tests. *Advances in Food Research*, 2, 219–258 (1949).
4. Harrison, S., and Elder, L. W. Some applications of statistics to laboratory taste testing. *Food Technol.*, 4, 434–439 (1950).
5. Highman, Arthur. How many samples make a valid test? *Western Industry*, May 1952.
6. Lockhart, Ernest E. Binomial systems and organoleptic analysis. *Food Technol.*, 5, 428–431 (1951).
7. Moncrieff, R. W. *The Chemical Senses.* 1944. John Wiley and Sons, New York, N. Y., pp. 1–424, especially p. 54.
8. Mood, A. M. *Introduction to the Theory of Statistics.* 1950. McGraw-Hill Book Co., New York, N. Y.
9. Neyman, J. *First Course in Probability and Statistics.* 1950. Henry Holt and Co., New York, N. Y.
10. Peryam, David R., and Swartz, VeNona W. Measurement of sensory differences. *Food Technol.*, 4, 390–395 (1950).
11. Roessler, E. B., Warren, J., and Guymon, J. F. Significance in triangular taste tests. *Food Research*, 13, 503–505 (1948).
12. Wood, Eric C. Organoleptic tests in the food industry. III. Some statistical considerations in organoleptic tests. *J. Soc. Chem. Ind.*, 68, 128–131 (1949).

THE SELECTIVITY OF ANETHOL AND THYMOL MEDIA FOR LACTIC ACID BACTERIA[a]

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There is a great need among food microbiologists for a selective medium to favor the growth of the lactic acid bacteria and, at the same time, to inhibit or prevent the growth of all other microorganisms. Approaches to this ideal have been made recently by descriptions of a liver infusion, sorbic acid medium by Vaughn and Emard *(19)* and Emard and Vaughn *(5)* and "Tween 80" media by Rogosa, Mitchell, and Wiseman *(16, 17)* and Evans and Niven *(6)*. However, it was hoped that other, even better media might be developed for the lactic acid bacteria by a study of the selective inhibitory effects of essential oils or their components.

It is known that different essential oils have varying antimicrobial properties [Anderson, Esselen, and Handelman *(1)*; Bachmann *(2)*; Blum and Fabian *(3)*; Corran and Edgar *(4)*; Fabian, Krehl, and Little *(7)*; Galloway *(8)*; James *(9)*; Kosker, Esselen, and Fellers *(10)*; Potter (15); Webb and Tanner *(20)*; and others]. As early as 1857 Pasteur *(14)* pointed out the possible use of the essential oil of onions for preventing the development of yeasts and obtaining a pure lactic acid fermentation because of the greater tolerance of the bacteria for that essential oil. Anethol and thymol, the major components of two of these essential oils, fennel and thyme, respectively, were observed by Molina and Merzari *(12)* to prevent the growth of film-forming yeasts on olive brines without undue disruption of the desirable lactic acid fermentation. They also found that some cultures of *Aerobacter aerogenes* were quite resistant to the inhibitory effects of anethol *(11)*. Later, on the basis of additional studies *(13)* they suggested the use of 3 or 4 drops of anethol, placed on the inside of a petri dish lid, as an aid in the isolation of lactic acid bacteria from olive brines highly contaminated with molds and yeasts. These observations indicated that both anethol and thymol could be incorporated in conventional bacteriological media to favor the growth of lactic acid bacteria (species of *Lactobacillus*, *Leuconostoc*, and *Streptococcus*) and inhibit other microorganisms. Promising exploratory experiments by the present authors were followed by a more detailed investigation of the selective inhibitory properties of these two compounds in various media. The results of this study are described below.

EXPERIMENTAL METHODS AND RESULTS

Basal media used to test the inhibitory effects of anethol and thymol were a glucose, tryptone medium (5 g. glucose and 10 g. Bacto-tryptone per liter) and liver infusion *(18)*, either as broths or with agar. The glucose agar (Bacto dextrose tryptone agar) was a

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Difco product. The liver infusion was prepared with 2% agar. All media were sterilized at 15 p.s.i.g. for 15 minutes.

The c. p. anethol and thymol used were obtained from the Eastman Kodak Co. Both compounds are considerably more soluble in organic solvents than in water. Therefore, they were diluted ten-fold with 95% ethanol before use in the media. The 2 stock solutions were stored at room temperature. The anethol was kept in a dark bottle because of its sensitivity to light. At a given concentration there was a significant reduction in inhibition of the test cultures when the compounds were added to the media before sterilization. Therefore, they were introduced after the media had been heated. In the case of the agar media they were added and mixed when the temperature was approximately 45° C.

A total of 108 different cultures including 2 genera of actinomycetes, 15 genera of bacteria, 15 genera of molds, and 6 genera of yeasts were used as test organisms. The names of these genera together with the number of cultures and species of each genus are:

Genera Tested

15 genera of bacteria
(66 cultures representing 43 species)

Acetobacter	3 cultures, 3 species
Aerobacter	4 cultures, 2 species
Azotobacter	1 culture, 1 species
Bacillus	19 cultures, 8 species
Corynebacterium	2 cultures, 2 species
Escherichia	5 cultures, 2 species
Lactobacillus	11 cultures, 6 species
Leuconostoc	3 cultures, 2 species
Microbacterium	2 cultures, 1 species
Propionibacterium	2 cultures, 1 species
Pseudomonas	3 cultures, 3 species
Salmonella	3 cultures, 3 species
Sarcina	2 cultures, 2 species
Shigella	2 cultures, 2 species
Streptococcus	4 cultures, 4 species

15 genera of molds
(27 cultures representing 27 species)

Absidia	1 culture, 1 species
Aspergillus	7 cultures, 7 species
Botrytis	1 culture, 1 species
Cephalothecium	1 culture, 1 species
Chaetomium	1 culture, 1 species
Fusarium	2 cultures, 2 species
Gliocladium	1 culture, 1 species
Memnoniella	1 culture, 1 species
Myrothecium	1 culture, 1 species
Neurospora	1 culture, 1 species
Paecilomyces	1 culture, 1 species
Penicillium	6 cultures, 6 species
Rhizopus	1 culture, 1 species
Scopulariopsis	1 culture, 1 species
Trichoderma	1 culture, 1 species

6 genera of yeasts
(11 cultures representing 9 species)

Candida	3 cultures, 3 species
Hansenula	1 culture, 1 species
Pichia	1 culture, 1 species
Saccharomyces	2 cultures, 2 species
Torulopsis	2 cultures, 1 species
Zygosaccharomyces	2 cultures, 1 species

2 genera of actinomycetes
(4 cultures representing 4 species)

Nocardia	2 cultures, 2 species
Streptomyces	2 cultures, 2 species

The cultures which showed a marked resistance to either anethol or thymol will be identified as to species.

The effect of concentration of anethol and thymol. The effect of concentration of anethol and thymol on growth of lactic acid bacteria was first determined in glucose tryptone broth. For this purpose, 100 ml. portions of the medium contained in 250 ml. cotton-plugged Erlenmeyer flasks were sterilized. Then the appropriate amounts of anethol or thymol were added to different portions of the medium. (Anethol was tested in concentrations of 0.01, 0.05, 0.10, 0.20, and 0.30% whereas the more reactive thymol was used in concentrations of 0.01 to 0.05% increased in increments of 0.01%). After inoculation with 0.1 ml. of culture the test media were incubated at 30° C. for 3 days. The experiments indicated that 0.2% anethol was sufficient to prevent growth of the test cultures of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* in the glucose tryptone broth. Growth was retarded with 0.10% anethol but apparently was uninhibited when the concentration was reduced to 0.05%. In comparison, a concentration of 0.03% thymol was sufficient to almost completely prevent the growth of the test cultures under the conditions of this experiment. However, when the concentration of thymol was reduced to 0.02% the 3 *L. plantarum* cultures grew well and the two *L. mesenteroides* strains still were inhibited slightly. The latter also grew well when the concentration of thymol was further reduced to 0.01%.

Glucose tryptone broth had been used because of its simple composition and ease of preparation. However, it was thought desirable to test liver infusion broth, a medium known to support species of *Lactobacillus* extremely well, and to compare the effect of thymol on growth when using this more productive medium. Thymol was chosen for the test because the concentrations in which it was to be used were within the range of its solubility in water (0.085 g./100 ml. at 20° C.) whereas pure anethol is almost insoluble in water. Furthermore, since relative growth was to be measured by use of the colorimeter, it was undesirable to use the anethol-alcohol mixture required because the higher concentrations caused a turbidity to appear in the medium.

The experiment was conducted in screw-cap 250 x 25 m.m. tubes because they could be used in the colorimeter and the per cent light transmission read directly. Accordingly, two series of tubes were prepared: one contained 25 ml. of glucose broth and the other contained 25 ml. of liver infusion per tube. Thymol was added in concentrations of 0.01, 0.015, 0.02, 0.025, and 0.03%, respectively, to each medium. Then each tube in both series was shaken, placed in the colorimeter and the deviation it produced from the tube containing the reference solution (100% transmission) was recorded. Both media were then inoculated with 0.1 ml. aliquots of the culture of *L. plantarum* grown in liver infusion broth. At time of use the inoculum contained 4.8 x 10^7 cells per 0.1 ml. The tubes were incubated at 30° C. for 100 hours. The differences in per cent light transmission resulting from differences in growth were taken at varying intervals.

Use of liver infusion broth did not increase the resistance of the *L. plantarum* culture to thymol. However, at a given concentration of thymol, more turbidity was produced earlier in the liver infusion than in the glucose tryptone broth. In the absence of any increased resistance to thymol by *L. plantarum* grown in the thymol, liver infusion medium, it was decided to make all additional tests with the glucose tryptone basal medium because of the ease of its preparation as compared with the liver medium.

To determine whether agar influenced the inhibition caused by anethol and thymol previously noted in liquid media, a series of tests were made with glucose (dextrose) tryptone agar (Difco). For this purpose various amounts of anethol and thymol were aseptically transferred to sterile petri dishes. Then exactly 20 ml. of sterile glucose tryptone agar were added and the contents thoroughly mixed. After solidification of the agar, the plate was marked into 4 sectors with a wax pencil. Then the agar in each sector was spot inoculated with a loopful of one of 4 organisms; *L. plantarum*, *Bacillus macerans*, *Pichia membranaefaciens*, or *Aspergillus fumigatus*. The plates then were incubated at 30° C. for 3 days.

It was observed that although *L. plantarum* could grow in the presence of 0.09% anethol uninhibited growth did not occur until the amount of anethol was reduced to 0.08% or less. In the case of thymol the maximum concentration tolerated by *L. plantarum* was 0.025% and partial inhibition still was evident at a concentration of 0.02%. At this latter concentration the mold *A. fumigatus* grew feebly. Therefore, it was apparent that selection of the concentrations of these compounds to be used in a medium designed to be selective for the lactic acid bacteria would be very critical.

The effect of pH on inhibition by anethol and thymol. The pH of the medium has been shown to affect the selectivity of the liver infusion-sorbic acid media described by Emard and Vaughn *(5, 19)*. Therefore, the effect of the pH value on the inhibitory power of anethol and thymol also was investigated. To determine this effect flasks of glucose tryptone agar were adjusted to the desired pH values and sterilized as before. Then, either 0.06% anethol or 0.015% thymol was added to the flasks of sterile medium. After thorough mixing, the 2 test media were distributed in petri dishes. The test media then were inoculated with the same 4 test cultures and incubated for 3 days at 30° C.

Inhibitory effects of both anethol and thymol were increased as the pH value of the glucose tryptone agar was decreased. The lowest pH values which favored the growth of *L. plantarum* under the conditions of this experiment appeared to be 6.2 and 6.4 in the anethol and thymol media, respectively.

The inhibition of microorganisms other than lactic acid bacteria. If an anethol or a thymol medium was to be useful for selective growth of lactic acid bacteria either one would have to inhibit all other microorganisms. As a final test of the selective action of these 2 compounds each of the 108 cultures of actinomycetes, bacteria, molds, and yeasts listed above were grown on glucose tryptone agar which contained either 0.08% anethol or 0.02% thymol. The final pH values of both test media were 6.7. Each petri dish of

test medium was streaked with a single culture. The same culture also was grown on plain glucose tryptone agar as a control. The inoculated media were incubated at 30° C. for 3 days.

None of the actinomycetes or yeasts grew on the media containing anethol or thymol and none of the molds grew on the medium with anethol. However, the 3 cultures *A. fumigatus, Aspergillus terreus,* and *Paecilomyces varioti* grew on the thymol medium approximately half as well as they did on the thymol-free control agar.

As shown in Table 1 all of the lactic acid bacteria tested grew in the presence of either 0.08% anethol or 0.02% thymol although marked inhibition was noted with some cultures. It is also interesting that a number of other bacteria grew extremely well in both of these media. The coliform bacteria and species of *Pseudomonas* were especially active. (In a subsequent test these 2 groups of bacteria still were uninhibited when the amount of thymol was increased to 0.25%, the concentration which markedly inhibited most of the lactic acid bacteria).

DISCUSSION

These observations indicated that if media with anethol or thymol were to be of value for selective isolation of lactic acid bacteria they had to be restricted in use to conditions where these bacteria predominated the microbial populations. Tests of this speculated restricted use were made in the field with samples of citrus fruit wash waters and normal fermenting cucumber brines and in the laboratory with various dehydrated vegetables. The media were of no value for detecting lactic acid bacteria in the majority of the dehydrated vegetable samples because the populations were not predominantly lactic acid bacteria. *Aerobacter, aerogenes* types predominated the microbial populations

TABLE 1

Resistance of various bacteria to 0.08% anethol and 0.02% thymol

Species	No. of strains	Glucose tryptone agar plus		
		Control	0.08% anethol	0.02% thymol
		Relative growth [1]		
Aerobacter aerogenes	3	3	3	3
Aerobacter cloacae	1	3	3	3
Azotobacter agile	1	3	1	1
Baccillus cereus	2	3	3	1
Bacillus coagulans	1	3	3	1
Bacillus polymyxa	1	3	3	1
Escherichia coli	4	3	3	3
E. coli, var. acidilactici	1	3	3	3
Lactobacillus acidophilus	1	3	3	2
Lactobacillus arabinosus	1	3	3	3
Lactobacillus casei	1	3	1	1
Lactobacillus pastorianus	1	3	2	2
Lactobacillus plantarum	5	3	2	2
Leuconostoc citrovorum	1	3	3	3
Leuconostoc dextranicum	1	3	2	2
Leuconostoc mesenteroides	1	3	3	3
Pseudomonas aeruginosa	1	3	3	3
Pseudomonas indoloxidans	1	3	2	2
Salmonella enteritidis	1	3	3	3
Shigella dysenteriae	1	3	2	2
Streptococcus faecalis	1	3	3	3
Streptococcus lactis	1	3	3	3
Streptococcus viridans	1	3	3	3
Streptococcus zymogenes	1	3	3	3

[1] 0 indicates no growth and 1, 2, and 3 indicate relative vigor of growth in 3 days at 30° C.

in the citrus wash waters to such an extent that the glucose tryptone agar with 0.02% thymol was useless for isolation of lactic acid bacteria. Yet, because the lactic acid bacteria were the most abundant types of organisms in the fermenting cucumber brines at the time of sampling, the thymol medium proved useful for their recovery. However, because similar results were obtained under identical field conditions and in the laboratory with plain liver infusion agar or other media useful for the growth of lactic acid bacteria, it was concluded that the use of either compound was too restricted to be of any real value. Therefore, the experiments were concluded without any investigation of the effects of either anethol or thymol on the species of *Clostridium*.

SUMMARY

Use of anethol and thymol in media to favor the growth of lactic acid bacteria has been described. The selective inhibition caused by either anethol or thymol was observed to be a function of concentration and was not enhanced by varying the pH value of the basal medium. The inhibition caused by both compounds increased as the pH of the medium was decreased.

Maximum concentrations which permitted growth of cultures of *L. plantarum* in glucose tryptone broth incubated at 30° C. for 3 days were 0.1% anethol and 0.03% thymol, respectively. When agar was used, the maximum concentrations tolerated did not change significantly, being 0.09% anethol and 0.025% thymol, respectively. The substitution of liver infusion as the basal medium did not change the maximum concentration of either compound still tolerated by *L. plantarum*.

Both anethol and thymol were more effective in inhibiting molds and yeasts than bacteria when used in concentrations that were not too inhibitory for the lactic acid bacteria. Some cultures, representing different species of the genera *Aerobacter, Bacillus, Escherichia, Pseudomonas, Salmonella,* and *Shigella* grew as well or better than the lactic acid bacteria in media containing 0.08% anethol and 0.02% thymol, respectively. Therefore, the prospective usefulness of either of these compounds as selective agents in media for isolation of lactic acid bacteria would be restricted to conditions where populations of these bacteria were predominant.

LITERATURE CITED

1. Anderson, E. E., Esselen, W. B., Jr., and Handelman, A. R. The effect of essential oils on the inhibition and thermal resistance of microorganisms in acid food products. *Food Research*, **18**, 40 (1953).
2. Bachmann, F. W. The inhibiting action of certain spices in the growth of microorganisms. *J. Ind. Eng. Chem.*, **8**, 620 (1916).
3. Blum, H. B., and Fabian, F. W. Spice oils and their components for controlling microbial growth. *Fruit Products J.*, **22**, 326 (1943).
4. Corran, J. W., and Edgar, O. H. Preservative action of spices and related compounds against yeast fermentation. *J. Soc. Chem. Ind.*, **52**, 149 (1933).
5. Emard, L. O., and Vaughn, R. H. Selectivity of sorbic acid media for the catalase negative lactic acid bacteria and clostridia. *J. Bacteriol.*, **63**, 487 (1952).
6. Evans, J. B., and Niven, C. F., Jr. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. *J. Bacteriol.*, **62**, 599 (1951).
7. Fabian, F. W., Krehl, C. F., and Little, N. W. The role of spices in pickled food spoilage. *Food Research*, **4**, 269 (1939).
8. Galloway, L. D. Essential oils as vapor phase antiseptics against mold fungi. *Perfumery Essent. Oil Record*, **43**, 359 (1952).

9. JAMES, L. H. Just how antiseptic are spices? *Food Inds.*, **3**, 524 (1931).
10. KOSKER, O., ESSELEN, W. B., JR., AND FELLERS, C. R. Effect of allyl isothiocyanate and related substances on the thermal resistance of *Aspergillus niger*, *Saccharomyces ellipsoideus*, and *Bacillus thermoacidurans*. *Food Research*, **16**, 510 (1951).
11. MERZARI, A. H., AND MOLINA, J. S. Influencia del aceite esencial de hinojo en el proceso fermentativo de aceitunas verdes "tipo español". II. Accion inhibitoria sobre el desarrollo de *Aerobacter aerogenes*. *Rev. Argentina Agron.*, **17**, 241 (1950).
12. MOLINA, J. S., AND MERZARI, A. H. Influencia del aceite esencial de hinojo en el proceso fermentativo de aceitunas verdes "tipo español". I. Accion inhibitoria sobre el desarrollo de las levaduras formadoras de velo superficial. *Rev. Argentina Agron.*, **16**, 234 (1949).
13. MOLINA, J. S., AND MERZARI, A. H. Influencia del aceite esencial de hinojo en al proceso fermentativo de aceitunas verdes "tipo español". III. Accion sobre los lactobacilos homo fermentativos. *Rev. Argentina Agron.*, **18**, 210 (1951).
14. PASTEUR, L. In Vallery-Radot, P. Memoire sur la fermentation appelée lactique. Oeuvres de Pasteur, **2**, 3, Paris, Masson et Cie. (1922).
15. POTTER, R. S. Antiseptic value of flavors. *Flavours*, **2**, (1), 7 (1939).
16. ROGOSA, M., MITCHELL, J. A., AND WISEMAN, R. F. A selective medium for the isolation and enumeration of oral and fecal lactobacilli. *J. Bacteriol.*, **62**, 132 (1951).
17. ROGOSA, M., MITCHELL, J. A., AND WISEMAN, R. F. A selective medium for the isolation and enumeration of oral lactobacilli. *J. Dental Research*, **30**, 682 (1951).
18. VAUGHN, R. H. The acetic acid bacteria. *Wallerstein Lab. Comm.*, **5**, (14), 5 (1942).
19. VAUGHN, R. H., AND EMARD, L. O. Selectivity of sorbic acid media for catalase-negative lactic acid bacteria and obligate, sporulating anaerobes. *Bacteriol. Proc.*, **1951**, 38 (1951).
20. WEBB, A. H., AND TANNER, F. W. Effect of spices and flavorings on the growth of yeasts. *Food Research*, **10**, 273 (1945).

DECOMPOSITION OF STARCH IN SAUSAGE PRODUCTS

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In many countries, limitations on the use of additives in sausage products are subjects of legislation. Some such ingredients, for instance potato flour, are necessary or at least desirable as binders in certain sausage products. Prerequisite to legislation, there should be reliable analytical methods to supply the controls that make possible the enforcement of laws on additives. In this paper the decomposition of starch and factors affecting it were investigated. Most of the products contained starch derived from potato flour, but there were also sausages in which milling products of wheat, rye, and barley formed the ingredients which were analyzed.

EXPERIMENTAL

The content of starch was determined according to the well-known method of Grossfeld *(2)* (slightly modified). This method gives reproducible results if prolonged treatment with the alcoholic KOH is avoided (45 min. by gentle boiling is applied) and the polarimeter-readings are made immediately or at least not more than 30 min. after dissolving the starch in HCl. The correctness of the method was ascertained by analyzing potato flours with known contents of starch, determined indirectly. Results of this method were compared with those obtained by gravimetric determination of the separated starch after due allowance for the water, ash, and protein content of the crude starch. This generally contained 10% moisture, 25–50% ash, ½–5% protein. The results agreed very well (Table 1).

TABLE 1

Comparison of polarimetric and gravimetric methods of analysis of starch content

Product examined	Content of starch, % according to	
	Polarimetric method	Gravimetric method
Beef	0.12	0.18
Veal, from newly born calves	0.6	0.6
Horse meat, frozen	0.6	0.7
Horse meat, frozen	0.4	0.4
Bologna sausage (Falukorv)	2.6	2.5
Bologna sausage (Falukorv)	2.1	2.2
Bologna sausage (Falukorv)	0.6	0.7
Bologna sausage, thick (Kokt Medwurst)	2.4	2.3
Bologna sausage, 2nd quality (Bräckkorv)	3.3	3.1
Fresh sausage, finely chopped (Rå fläskkorv)	2.8	3.0
White pepper	56.5	56.4

From preliminary investigations it became evident that the starch was decomposed in sausages which had been pasteurized or otherwise treated by heat; the starch had been converted into a paste. Likewise the starch was decomposed in sausages containing starch products such as cooked potatoes and cooked pearl barley. In these products, the starch had turned gelatinous prior to the addition to the minced or chopped meat. On the other hand, native starch is not attacked. This was easily demonstrated in the case of starch in

fresh sausage composed of finely chopped pork, veal, beef, and potato flour. Here the starch remained intact until the sausage became spoiled.

How is the decomposition of the starch to be explained? When added to fresh meat in a cooked (gelatinous) state it is likely to be attacked by the α-amylase present in meat. The effect of the enzyme is accelerated by salt. In summer sausage, acid hydrolysis and action of microorganisms must be considered, too. When native starch is heated along with the meat ingredients in the sausage, however, gelatinization of the starch, inactivation of the meat enzymes, and the destruction of vegetative bacteria will almost coincide. In consequence, it is to be expected that rate of heating, width of the sausages, and final temperature attained will play roles in the decomposition of starch during and after processing. Hence, samples were taken from various types of sausages during different stages of processing under practical conditions in the plant, and during storing as well, for analyzing the content of starch. For these experiments homogenization was carefully accomplished and, in addition, 5 samples were collected from each stage and mixed. The weight of potato flour, etc., the weight of the whole lot of sausage, and the shrinkage at each stage was controlled to enable calculating the starch content on an equal basis. Thirty-eight lots were examined, the results of which are summarized in Table 2. Also, the reduction power of extracts of the sausages was tentatively determined according to the method of Willstätter-Schudel, modified by Myrbäck *(4)*.

TABLE 2

Changes in starch content during processing and storage of various kinds of sausage products

All data are calculated on a fresh (raw) weight basis. *Temp. in core* means temperature in core at the end of the smoking or cooking period. *Finished* means cooked, cooled in a shower-bath (except Nos. 31–32) and stored over night in the cooler.

Storage temperature, unless otherwise stated was 6–10° C.

Observations. Products Nos. 1–26 were emulsion sausages, to which 4–4½% potato flour had been added (with the exception of No. 4), calculated on a final weight basis. According to Swedish law, 5% potato flour is allowed in the finished product.

Products Nos. 1–4 were finely chopped fresh sausages as opposed to the English and American types of pork sausage, which are usually coarsely chopped. In sausage No. 4, 35% cooked potatoes were added instead of potato flour. Meat ingredients: pork, veal and beef in Nos. 1–3, pork and beef in No. 4. Content of protein was about 8–10%, fat 20–25%, water 60–62%.

Nos. 5–26 were finely chopped, smoked and cooked sausages containing pork, beef, and sometimes veal (as *e.g.* in wieners). Bologna, 2nd and 3rd quality, and luncheon sausage, 2nd quality, also contained hearts. Unless otherwise stated, the smoking lasted between 1½ and 2 hrs.; smoking temperature was 60–70° C. When smoking wieners, 80° C. was applied at the end of the period. Cooking time varied between 10 min. (wieners) and 1 hr. (thick bologna). Cooking temperature was kept between 75–80° C. Thus, the temperature in the core of the sausage had attained at least 70° C. at the end of the cooking period. Content of protein in these sausages was about 10%, fat 25%, water 55–60%.

Nos. 27–30 were smoked (but not cooked) coarsely chopped sausages, containing beef and pork. Content of water was about 50%.

Nos. 31–33 were cooked but not smoked products constituting a mixture of 2 parts cooked meats (beef, pork, hearts, lungs, kidneys) and 1 part cooked pearl barley, each kind separately cooked.

The liver pastes (Nos. 34–36) contained *i.a.* 40% pig liver, 40% pork, and 2% wheat flour. They were cooked for 2½ hrs. at 85° C. in moulds holding 2½ kg.

No. 37 and 38 contained 44% pig blood, 10% syrup, and 20% rye flour. The sausage was cooked in horse casings (diam. 70 mm. and wider) for 2¼ hrs. at 80° C., the black pudding was cooked for 2¾ hrs. at 100° C. in cylindric moulds (diam. 125 mm.) holding 3 kg.

The diameter of sausages Nos. 5–9 was 65 mm.; Nos. 10–11 and 14–15: 52 mm.; Nos. 12–13: 43–46 mm.; Nos. 16–20: 32–35 mm.; Nos. 21–22: 21–23 mm.; Nos. 23–24: 20 mm.; Nos. 25–26: 18–20 mm.; Nos. 27–28: 65 and 50 mm.; Nos. 29–30: 32–37 mm.; Nos. 31–32: 38 mm. and greater.

The length of the sausages Nos. 16–20 was 15 cm.; Nos. 21–22: 19 cm.; Nos. 23–24: 15 cm.; Nos. 25–26: 6 cm.; Nos. 27–28: 40 and 60 cm.; Nos. 29–32: 15 cm.
The Swedish names of the products appear in parentheses.

Lot No.	Product	Stage of processing	Starch content, %	Reduction power, calculated as % maltose
1	Fresh sausage (Rå fläskkorv) pH of sausage: 5.8; 5.4; and 5.0, respectively	fresh	3.3	1.3
		stored 2 days in brine*	3.4	0.9
		stored 3 days in brine*	3.4	0.3
2	Fresh sausage	fresh	3.4	1.4
		stored 1 day (dry)	3.3	1.4
		stored 1 day in brine*	3.3	0.8
3	Fresh sausage	fresh	3.2	1.2
		stored 1 day (dry)	3.1	1.2
		stored 1 day in brine**	3.1	0.8
4	Potato sausage, fresh (Värmlandskorv) pH of sausage: 6.0; 6.0; and 5.9, respectively	fresh	4.9	2.3
		stored 1 day in brine*	2.7	2.4
		stored 2 days in brine*	2.8	2.1
5	Bologna, thick (Kokt medwurst)	fresh	3.8	1.3
		smoked	3.6	1.7
		finished	2.5	1.5
		stored 7 days	2.1	2.1
6	Bologna, thick Smoking {time: 125 min. {temp. in core: 54° C.	fresh	3.0	1.8
		smoked	3.0	1.6
		finished	0.5	3.4
		stored 7 days	0.3	3.2
7	Bologna, thick Smoking {time: 120 min. {temp. in core: 52° C.	fresh	4.1	1.8
		smoked	3.0	1.9
		finished	3.0	2.4
		stored 7 days	2.6	2.1
8	Bologna, thick Smoking {time: 120 min. {temp. in core: 55° C. Cooking {time: 50 min. {temp. in core: 72° C.	fresh	3.8	1.3
		smoked	3.3	1.2
		finished	2.7	1.8
		stored 7 days	2.0	2.2
9	Bologna, thick	fresh	3.4	1.6
		smoked	3.3	1.9
		finished	2.8	1.8
		stored 7 days	2.7	2.1
10	Bologna (rings) (Falukorv)	fresh	3.9	1.3
		smoked	3.7	1.3
		finished	3.7	1.4
		stored 7 days	3.4	1.5
11	Bologna (rings) Smoking {time: 150 min. {temp. in core: 60° C.	fresh	3.6	1.8
		smoked	3.3	1.6
		finished	3.4	1.5
		stored 7 days	3.1	1.8
12	Bologna (rings), 2nd quality (Bräckkorv)	fresh	3.1	2.0
		smoked	3.1	2.2
		finished	3.0	2.4
		stored 8 days	2.7	2.5
13	Bologna (rings), 2nd quality Smoking {time: 120 min. {temp. in core: 59° C.	fresh	3.9	1.7
		smoked	4.0	2.2
		finished	3.9	2.1
		stored 7 days	3.8	2.4

Lot No.	Product	Stage of processing	Starch content, %	Reduction power, calculated as % maltose
14	Bologna (rings), 3rd quality (Hackkorv)	fresh	3.5	—
		finished	0.3	—
15	Bologna (rings), 3rd quality	fresh	3.3	1.6
		finished	0.1	4.5
16	Luncheon sausage (Frukostkorv)	fresh	3.4	1.2
		smoked	2.7	1.4
		finished	2.7	1.4
		stored 7 days	2.4	1.5
17	Luncheon sausage	fresh	3.4	1.8
	Smoking {time: 90 min. / temp. in core: 60° C.	smoked	2.7	1.8
		finished	2.6	1.9
		stored 5 days	2.1	2.5
18	Luncheon sausage, thick, 2nd quality (Knackwurst)	fresh	3.3	1.3
		smoked	2.9	2.0
	Smoking {time: 120 min. / temp. in core: 51° C.	finished	1.1	3.1
		stored 7 days	0.8	3.6
19	Luncheon sausage, thick, 2nd quality	fresh	3.7	0.9
		smoked	3.7	1.4
	Smoking {time: 120 min. / temp. in core: 52° C.	finished	1.4	2.4
		stored 6 days	1.4	3.3
20	Luncheon sausage, 2nd quality	fresh	3.5	1.5
	Smoking {time: 90 min. / temp. in core: 60° C.	smoked	3.2	1.5
		finished	2.9	1.9
		stored 7 days	2.2	2.1
21	Frankfurters (Varmkorv)	fresh	4.0	1.7
		smoked	3.7	1.8
		finished	3.7	1.8
		stored 7 days	3.6	1.8
22	Frankfurters	fresh	3.7	2.1
	Smoking {time: 90 min. / temp. in core: 62° C.	smoked	3.6	2.7
		finished	3.1	3.2
		stored 6 days	3.1	3.7
23	Wieners (Wienerkorv)	fresh	3.5	1.8
		smoked	2.9	2.2
		finished	2.9	1.9
24	Wieners	fresh	3.7	0.9
	Smoking {time: 90 min. / temp. in core: 72° C.	smoked	2.4	1.6
		finished	2.5	1.5
	Cooking {time: 10 min. / temp. in core: 75° C.	stored 7 days	2.4	1.5
25	Small wieners (Prinskorv)	fresh	2.9	1.0
	Smoking {time: 90 min. / temp. in core: 63° C.	smoked	1.5	1.7
		finished	1.6	1.6
		stored 7 days	1.4	2.2
26	Small wieners	fresh	3.0	1.5
	Smoking {time: 90 min. / temp. in core: 64° C.	smoked	1.9	2.2
		finished	2.0	2.0
	Cooking {time: 10 min. / temp. in core: 75° C.	stored 8 days	1.8	2.2

Lot No.	Product	Stage of processing	Starch content, %	Reduction power, calculated as % maltose
27	Semidry Cervelat with cooked potatoes (Hushållsmedwurst) Drying and smoking {time: 4 days / temp.: 25–30° C. pH of sausage: 5.9; 5.9; 4.9; 4.6; and 4.8, respectively Storage temp. 15° C.	fresh	2.4	2.9
		dried 15 hrs.	0.8	3.3
		smoked	0.7	4.8
		stored 10 days	0.6	4.6
		stored 15 days	0.7	4.8
28	Semidry Cervelat with cooked potatoes Drying and smoking {time: 4½ days / temp.: 23–25° C. pH of sausage: 5.8; 5.0; and 5.0, respectively Storage temp. 15° C.	fresh	2.4	2.9
		smoked	1.6	3.3
		stored 14 days	1.5	3.8
29	Smoked fresh sausage with cooked potatoes and cooked pearl barley (Isterband) Drying and smoking {time: 30 hrs. / temp.: 20–40° C. pH of sausage: 6.0; 5.8; 5.4; 5.1; and 4.5, respectively	fresh	6.7	1.6
		dried 14 hrs.	6.3	2.3
		smoked	6.3	2.3
		stored 1 day	6.1	2.4
		stored 8 days	5.4	2.8
30	Smoked fresh sausage with cooked potatoes and cooked pearl barley Drying and smoking {time: 30 hrs. / temp.: 20–40° C. pH of sausage: 5.9; 4.9; 4.5; 4.4; and 4.7, respectively	fresh	6.7	2.0
		smoked	6.0	2.3
		stored 2 days at 18° C.	5.6	2.6
		stored 4 days at 18° C.	5.5	2.5
		stored 7 days at 8° C.	5.8	2.8
31	Cooked sausage with cooked pearl barley (Stångkorv) pH of sausage: 6.5; 6.3; and 5.9, respectively	finished	7.3	1.7
		stored 1 day at 8° C.	6.8	1.7
		stored 3 days at 8° C.	6.7	2.3
32	Cooked sausage with cooked pearl barley pH of sausage: 6.1; 5.2; and 4.7, respectively	finished	7.5	2.2
		stored 2 days at 18° C.	6.7	2.6
		stored 4 days at 18° C.	6.6	2.6
33	Product composed similar to the preceding item, not in casings but canned for 90 min. at 123° C. in 1 kg. cans (Pölsa)	mixed cooked ingredients	7.7	1.7
		canned	7.4	2.6
34	Liver paste (Leverpastej) (meat ingredients chopped in the fresh state)	fresh	2.7	5.7
		cooked	1.9	7.1
		stored 6 days at 9° C.	1.8	6.9
35	Liver paste (meat ingredients chopped in the fresh state)	fresh	1.9	4.9
		cooked	1.5	6.9
		stored 7 days at 8° C.	1.4	6.8
36	Liver paste (meat ingredients precooked before being chopped)	fresh	2.1	5.7
		cooked	1.7	6.8

Lot No.	Product	Stage of processing	Starch content, %	Reduction power, calculated as % maltose
37	Black sausage	fresh	10.8	4.7
		cooked	8.9	6.9
38	Black pudding	fresh	10.8	4.7
		cooked	9.5	7.5

* Concentration of brine: 4%.
** Concentration of brine: 3 and 4%. No different effect was observed in these brines.

CONCLUSIONS

As has been mentioned, the native starch in the fresh sausages Nos. 1–3 was not (or practically not) affected, not even after storage. Of course, the reduction power would diminish when storing in brine, due to dialysis of reducting matters through the casing (hog casing).

In fresh sausage No. 4, however, the starch of the cooked potatoes decomposed to a marked degree, from 4.9% to 2.7%.

As to the bologna sausages, Nos. 5–15, the decomposition of the starch had gone particularly far in the thick ones. An extreme break-down had occurred in No. 6, less in Nos. 5, 7, and 8. As a rule, the starch was decomposed during cooking, although there were examples of a break-down during smoking, too (*cf.* No. 7). Evidently, disappearance of part of the starch occurred even during storage at relatively low temperatures (*cf.* Nos. 8, 5, and 7). In the thinner types of bologna, represented by Nos. 10–13, the decomposition of starch was insignificant. In the sausages Nos. 14 and 15, however, the break-down of the starch was extremely high, probably due to the enzymatic activity contributed by the organs present in this type of sausage.

The still thinner smoked and cooked sausages, represented by Nos. 16–26, showed a somewhat varying behavior with regard to starch decomposition. Thus, in Nos. 16 and 17 a significant break-down of starch occurred during the smoking process (from 3.4 to 2.7%), and, especially when considering No. 17, also during storage (from 2.6 to 2.1% in 5 days). On the other hand, the starch in Nos. 18–20 was mainly decomposed during the cooking process, due to a relatively low smoking temperature. In Nos. 18 and 20 a significant "disappearance" of starch occurred during storage.

In frankfurters and wieners (Nos. 21–24) the starch passed through the whole manufacturing process and storage without being seriously attacked. Yet, No. 24 was an exception insofar as a break-down of starch from 3.7 to 2.4% occurred during smoking. No doubt this was on account of the high smoking temperature, which caused 72° C. in the core at the end of the smoking period. In sausages Nos. 25 and 26 the percentage of starch added was significantly lowered (1-1½%) during smoking, probably for the same reason as in No. 24, but no marked change was observed during storage.

The reduction power of the sausages Nos. 4–26 was inversely proportional to the starch content. There were, however, exceptions. In these cases the reduction power, calculated as maltose, as a rule was a little lower than was to be expected from the decomposition of the starch.

In sausages Nos. 27 and 28 a strong break-down of the starch, deriving from the cooked potatoes, occurred. Although of a different type than sausage No. 4, the source of starch was the same. Thus, the results were similar, too.

The loss of starch was exactly compensated by a corresponding gain in "maltose".

Sausages Nos. 29 and 30, containing cooked potatoes and cooked pearl barley, showed a moderate decrease in starch during smoking. During the storage period, however, the decomposition of starch was greater. There are three factors which may contribute to an explanation of these matters: 1) time is required to attack the cooked pearl barley; 2) pH is changed from about 6 to 4½; hence hydrolysis must be considered; 3) growth of bacteria is favoured by time; a break-down caused by bacteria may also occur.

In sausages Nos. 31 and 32 a moderate lowering of the starch content, between ½ and 1%, occurred during storage, probably for the same reasons (except the first one) as those mentioned in discussing the two preceding products containing pearl barley. In the present type of sausage the ingredients were cooked before mincing and mixing. Thus, the development of bacteria was due to infection during these processes and filling as well as to the infection by the casings. During the canning process only a small change of the starch content was observable (*cf.* No. 33).

To the liver pastes, Nos. 34–36, 2% wheat flour was added. Additionally, as a source of carbohydrate, the presence of glycogen must be assumed, although most of it usually is broken down before processing. During cooking the starch content was lowered ½–1%, whilst the reduction power, calculated as maltose, increased 1–2%. Thus, reducing substances other than maltose were formed, too. The decomposition of starch was shown to go farther in the internal parts of the paste than in the outer ones, evidently due to a more rapid heat inactivation of the amylase in the latter parts. For instance, in liver paste No. 34, an outer layer constituting 13.7% of the total paste contained 2.53% starch and an inner layer constituting 14.9% had a starch content of 1.95%, calculated on the same basis.

When comparing black sausage and black pudding, Nos. 37 and 38, both of which had the same original composition, the break-down of starch was greater in the former than in the latter. The reason for this is very likely the lower cooking temperature of the sausage (80° C.), enabling the amylases in rye and in blood to act during a relatively longer period or until they become heat inactivated. On the other hand, part of the reducing substances (glucose, maltose, etc.) are dialysed through the casings. No difference between horse casings and cellophane casings was observed in this respect. So the reduction power of the sausage, in spite of a more far-reaching decomposition of starch in the sausage than in the pudding, turned out to be less than in the black pudding, which was cooked at 100° C. in closed moulds. Here again, as was the case with liver paste, the starch was broken down to a higher extent in the center parts of the black pudding than in its outer layers. Thus, 12.7% starch was found in an outer layer constituting 5.5% of the pudding as compared to 9.1% starch in a core constituting 25% of the total pudding. The reduction power, calculated as maltose, was 7.3% and 9.1% respectively. The content of "maltose" in the rye flour used was 4.1% as compared to 4.7% in the fresh black pudding.

Observations on the decomposition of starch. From the discussion so far, it will be noted that a common feature of the decomposition of starch in sausage products is the relation to heat treatment. It seems certain, that 1) gelatiniza-

tion of the starch is a prerequisite for being acted upon, and 2) degradation by amylases are the most important factors involved in the break-down of the starch. Generally, the processing time, during which the temperature is favourable for growth of bacteria, is very short. Moreover, smoking will retard the development of microorganisms. Thus, a degradation of starch by microorganisms may only be considered in some cases during processing (Nos. 27–30) and during storage (Nos. 29–32). In the products Nos. 27–32 the conditions for acid hydrolysis were present. On the other hand, the action of animal amylases is of minor importance at low pH. Whether or not bacterial action is of any importance in the decomposition of starch during storage of the cooked sausages Nos. 5–26 is uncertain, practically all vegetative microorganisms being killed during the cooking process. An approach to this matter was made by examining the number of microorganisms in the sausages after processing and at the end of the storage period. The number of microorganisms in the fresh sausages and in the various stages of processing was also determined but seems to be of no particular importance in this connection. The result was an almost invariable number of microorganisms growing on broth agar, the magnitude being 10^3 to 10^4 per gram (apart from No. 5, see below), both after cooking and at the end of the storage period (7 days). In some cases even a lesser number of microorganisms was observed after storing than after cooking. In particular, the number of microorganisms in sausage No. 6 with highly decomposed starch was $2 \cdot 10^3$ per gram after cooking and $1 \cdot 10^3$ after storage for 7 days, as opposed to the sausage No. 5 with moderately decomposed starch, the corresponding figures being $1 \cdot 10^2$ and $2 \cdot 10^5$. For comparison, the number of microorganisms in the sausages Nos. 27–28 and Nos. 29–30 growing on broth agar may be mentioned: in Nos. 27–28: $1.5 \cdot 10^7$ (fresh), $1 \cdot 10^7$ (smoked), and $7.5 \cdot 10^7$ (stored 14 days); in Nos. 29–30: $7 \cdot 10^5$ (fresh), $4 \cdot 10^8$ (smoked), and $3 \cdot 10^8$ (stored 7 days).

Thus, bacterial activity is likely to be of no importance during low temperature storage of smoked cooked emulsion sausages Nos. 5–26. A fact, which has not been regarded in full, is the variability of the quality of potato flour. Marked differences as to the viscosity of various lots of potato flour have been observed. With the exception of four of the sausage products investigated, one of which was No. 6, wherein the starch was highly decomposed during processing, potato flour of practically the same viscosity was used in manufacturing the sausages used in these experiments.

Alpers and Ziegenspeck *(1)* have also investigated the degradation of starch in sausage products. They stressed the autolysis of starch pastes as an explanation of the disappearance of starch in sausages. It may be that autolysis *is* responsible for the breakdown of starch under certain conditions and probably the quality of starch is of importance in this connection, but, when considering the very small changes of the starch content in sausages Nos. 9–13, 16, 19, and 21–26 during storing, autolysis must have played a minor part in starch degradation. These authors also concluded that enzymatic decomposition of starch must be of great importance. On the other hand, although the conclusions of these authors as to the break-down of starch by bacteria are in accordance with the findings in some fresh sausages (Nos. 27–32), they are not in accordance with the findings on smoked and cooked sausages Nos. 5–26.

Unfortunately, the blank test of the reduction power, *i.e.* the value of

reduction of the fresh sausages, cannot be considered as a constant, not even for the same type of sausage, as for example the Nos. 1–26 (except No. 4). Thus, the reduction power, calculated as maltose, fluctuated between 0.9 and 2.1%. Yet, 1½% seems to be a rather useful mean. About half of that was later obtained in a series of analyses of the reduction power by means of Fehling's solution, using phosphotungstic acid as the precipitant for protein. However, the same variations of the blank test as mentioned above were obtained. This was true both in fresh sausages and in beef, veal, or pork alone. The determination of the reduction power of the carbohydrates is affected *i.a.* by the amino acids present in meat. A striking example of a high blank test is that of liver paste (Table 2), owing to an extensive glycogenolysis and proteinolysis in liver tissue. A recent investigation of the influence of amino acids on the determination of reducing carbohydrates according to various methods is made by Iwainsky *(3)*.

SUMMARY

Thirty-eight different sausage products were investigated with regard to the decomposition of starch during processing and storage. Four fresh and 22 cooked and smoked products of the emulsion sausage type containing potato flour as an ingredient were examined. Further, 4 smoked, semidry sausages (summer sausages) containing cooked potatoes and cooked pearl barley, respectively, were investigated. Finally, 3 sausages containing cooked pearl barley (in which the meat ingredients and the barley were separately cooked before mixing), 3 liver pastes, 1 black sausage, and 1 black pudding were also included in the experiments.

Results are summarized in Table 2. Generally, the content of starch was markedly decreased during the cooking process when gelatinization of the starch occurred. Although no proof of amylase activity was given in these experiments, the gelatinized starch is likely to have been attacked by this enzyme. Non-gelatinized starch remained unattacked. Decomposition of starch during storage is of importance primarily in summer sausages and to some extent also in thick bologna. In the former products bacterial action and acid hydrolysis may play roles in the decomposition of starch.

The reduction power was increased as the starch was decomposed.

LITERATURE CITED

1. Alfers, K., and Ziegenspeck, H. Beiträge zur Beurteilung der Stärkebestimmungsverfahren. *Z. Untersuch. Nahr. Genussm.*, **45**, 163 (1923).
2. Grossfeld, J. Die Bestimmung der Stärke in Wurstwaren. *Z. Untersuch. Nahr. Genussm.*, **42**, 29 (1921).
3. Iwainsky, H. Über den Einfluss von Aminosäuren auf die Bestimmung reduzierender Kohlenhydrate. *Z. Lebensm. Untersuch. u. -Forsch.*, **100**, 173 (1955).
4. Myrbäck, K. Die Oxydation der Aldosen durch Hypojodit. V. *Svensk Kem. Tidskr.*, **51**, 206 (1939).

A DIRECT SURFACE AGAR PLATE LABORATORY METHOD FOR QUANTITATIVELY DETECTING BACTERIAL CONTAMINATION ON NONPOROUS SURFACES [a]

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Investigations concerned with quantitative detection of the bacterial contamination on surfaces are often impeded, because the presently available techniques are limited either in reliability or application to certain surfaces. The American Public Health Association *(2, 3)* recommends the cotton swab rinse method for examination of food and dairy utensils. However, this technique merely yields an index of the sanitary quality of utensils and is not a quantitative method *(4)*.

This investigation was undertaken to develop a direct surface agar plate (DSAP) method by which the bacterial contamination on nonporous surfaces could be quantitatively determined. This technique was needed to provide a means for comparison of commonly used recovery methods.

EXPERIMENTAL PROCEDURES

Direct surface agar plate (DSAP) method. DSAP determinations of the bacterial contamination on surfaces were performed by drawing a circle, the area of which was 4.0 sq. in. (diameter 2¼ in.) on the test surface employing a small Petri dish bottom as a template and a wax pencil. Sterile agar medium was pipetted into the circle in the ratio of 0.25 to 0.5 ml. of medium per sq. in. of surface. Any area may be employed in making such a determination, but the above ratio of medium to surface area sampled must be maintained. The agar was spread evenly over the area with the end of the pipette and was contained within the specified area by the wax pencil line. Upon hardening, the circle of agar was covered with a Petri dish lid containing a piece of moistened filter paper. The test object was then incubated in a high humidity chamber (approximately saturated atmosphere) at 35° C. for 16 to 18 hours. The moistened filter paper and humid atmosphere prevented the agar from dehydrating, and during incubation colonies developed. Following incubation, the preparations were removed from the incubator and the agar circles were flooded, dropwise, with 0.5% aqueous solution of 2,3,5-Triphenyl 2 H-tetrazolium chloride and permitted to stand at room temperature for 10 to 15 minutes or until all the colonies developed a deep red color. The stained preparations were dried in an oven at 65 to 70° C. for 10 to 15 minutes. Slow drying at room temperature was also found to be satisfactory. Organic soil or extraneous matter on the surface did not interfere with the specificity of the staining reaction. The stained colonies were counted by placing a glass grid over the dried preparation and observing it under a stereoscopic microscope (15 X) with a bright light directed onto the surface from a 45° angle.

Comparison of DSAP method to conventional plate count technique. The ability of the DSAP method to measure quantitatively surface contamination was determined by a comparison of the recovery of organisms by this method against a conventional plate count technique used to determine the numbers of organisms present in the contaminating inoculum.

Bacillus globigii spores [b] were suspended in sterile distilled water and filtered through Whatman No. 2 paper. After filtering, the spores were washed 5 times in sterile distilled

[a] This work was supported in part by the Federal Civil Defense Administration under Delegation No. 1 to the Department of Health, Education, and Welfare.

[b] Obtained in a concentrated "mud" form from Fort Detrick, Frederick, Maryland.

water by centrifugation, resuspended in sterile distilled water, heat shocked at 80° C. for 10 minutes and stored at 5° C. Just before use, an aliquot of the spore suspension was again heat shocked and diluted in sterile distilled water to yield the desired concentration. Final concentrations of viable spores per ml. were determined by preparing triplicate tryptose agar pour plates which were counted after 48 hours incubation at 35° C.

Micrococcus pyogenes var. *aureus* 209 was grown on brain heart infusion agar slants incubated at 35° C. and transferred daily to fresh slants. The growth from a 24-hour slant was suspended in sterile, buffered, dilution water *(1)* and adjusted to 90% transmittance at 620 mμ on a Coleman Junior spectrophotometer. The suspension was diluted 1–10,000 in reconstituted, sterile, skim milk (10% solids) to provide a protective colloid for the cells when spread and dried on the test surfaces. These suspensions contained approximately 5,000 to 13,000 viable cells per ml. as determined by triplicate platings in tryptone glucose extract agar which were counted after 48 hours incubation at 35° C.

Triplicate, vitreous china, salad dishes (plates 7 in. diameter) were contaminated by spreading 0.1 ml.-aliquots of the prepared bacterial suspensions over a 4 sq. in. area in the center of the dishes. Dishes contaminated with the spore suspensions were dried 10 minutes at conditions which ranged throughout the daily experiments from 70 to 85° F. and 21% to 55% relative humidity; dishes contaminated with micrococci were dried 10 minutes at 80° F. and 60% relative humidity.

DSAP determination on china surfaces coated with organic soils. Various spore-soil mixtures were prepared from the heat shocked aqueous spore suspension and used to contaminate china plates as described above. Spore-soil mixtures were such that a 0.1-ml. aliquot contained approximately 500 spores by plate count. Soils consisted of the following: distilled water, reconstituted nonfat dry milk (10% solids), 10% aqueous suspension of prepared cake mix, whole egg suspension, 10% sucrose solution, and melted (60° C.) hydrogenated vegetable shortening.

DSAP determinations on a variety of surfaces. Employing the procedures described above, triplicate squares of vitreous china, painted wood, unpainted wood, plastic, stainless steel, plastic food wrapping film, meat wrapping paper, paper picnic plates, and washed, white cotton fabric were contaminated by spreading 0.1-ml. aliquots of either a water suspension containing 0.5% Tween 80 or sterile skim-milk suspension of spores over an area of 4 sq. in. on the surface of each square. Unglazed porcelain was also used; but, because of the highly absorbent nature of the porcelain, a 1.0-ml. aliquot of suspension containing an equal number of spores was used as an inoculum.

RESULTS

The ability of the DSAP method to recover quantitatively *B. globigii* spores from china surfaces is shown in Table 1. Based on averages of triplicate determinations, the direct surface agar plate detected from 87.6% to 100.9% of the number of spores inoculated on the salad dishes. Coefficients of variation and the 95% tolerance limits for single determinations, as well as averages of triplicate determinations, were calculated for the DSAP and conventional pour plate methods. Tolerance limits can be stated as follows:

TABLE 1

Comparison of conventional plate count to the DSAP method for recovering Bacillus globigii spores and Micrococcus pyogenes var. aureus 209 from china surfaces

Bacillus globigii spores			*Micrococcus pyogenes* var. *aureus* 209		
Surface inoculum by plate count [1]	Recovery by DSAP method [1]	Per cent recovery	Surface inoculum by plate count [1]	Recovery by DSAP method [1]	Per cent recovery
113	99	87.6	670	569	84.9
103	101	98.0	720	507	70.4
120	115	95.8	1,060	1,030	97.2
122	112	91.8	1,330	1,069	80.4
113	108	95.6	970	494	50.9
123	111	90.2	640	513	80.1
109	110	100.9	960	924	96.2

[1] Each value represents the average from triplicate determinations.

if single determinations are made by the conventional plate count and by the DSAP method, using equal aliquots of the same suspension, the latter will detect from 84.7% to 103.5% of the number of spores present in the inoculum as determined by the plate count, 95 trials out of 100. Similarly, if 2 groups of 3 determinations are made by each method and their averages compared, as was actually done in the above experiments, the DSAP method will determine between 88.5% and 99.3% of the contamination, 95 trials out of 100.

In the experiments with micrococci (Table 1) quantitative recovery equivalent to that obtained with spores did not occur. Recovery of micrococci by the DSAP method ranged from 50.9% to 97.2% in the 7 experiments. Variation between triplicate counts within a single experiment cannot account for the entire range of recoveries obtained. This result infers that death of the cells during drying varied from experiment to experiment but not between replicates within a single experiment. Since the environmental conditions of temperature, humidity, inoculum size, and experimental time limit were rigidly controlled in all seven trials, it is likely that most of the variation was related to differences in susceptibility to drying among the individual cultures employed.

For a given experiment, ratios of single DSAP counts to single plate counts vary between limits which are dependent on the amount of death due to drying. Tolerance limits are directly proportional to the average percentage recovery for that experiment. Specifically, in 95 trials out of 100, the DSAP to plate count ratio will lie between 71% and 141% of the average percentage recovery obtained.

The average percentage recovery of the 7 experiments performed was about 80%, so that ratios of individual DSAP counts to plate counts would vary between .57 and 1.13 in such an average experiment, 95% of the time.

Using averages of triplicate determinations by each method, the 95% tolerance limits for their ratio are narrowed to 91% to 110% of the average percentage recovery in a given experiment. In an experiment with 80% recovery, such replicate ratios would vary between .73 and .88.

Comparison of the precision of the DSAP method with that of the conventional plate count technique revealed that the variation between replicate counts of both techniques was equivalent. The coefficient of variation was shown to be 7.1% in the spore determinations and 11.5% for the micrococcal experiments.

With water or milk suspensions of spores, application of the direct surface agar plate method to various surfaces revealed that recovery was dependent to a great extent upon the nature of the surface material. Surfaces containing toxic constituents or highly porous surfaces yielded low recovery rates. Table 2 shows the average per cent recovery of spores from various surfaces and reveals that recoveries of the type described for china were obtained only on stainless steel and plastic. However, it was not possible to determine from these experiments whether the low recovery on the other surfaces was due to death of the spores or to the fact that the agar medium was not accessible to spores sheltered in the minute cracks and fissures of the surface.

TABLE 2

Recovery of B. globigii spores from various surfaces by means of the Direct Surface Agar Plate method

Type of surface contaminated	Average per cent recovery[1] of surface inocula applied in:	
	Water 357–438 spores per 0.1 ml.	Milk 410–572 spores per 0.1 ml.
Glazed porcelain	91.5	97.0
Unglazed porcelain	71.3	84.0
Painted wood	0.0	0.0
Unpainted wood	0.0	0.0
Plastic	90.4	96.0
Stainless steel	90.6	94.0
Plastic wrapping film	45.3	59.1
Meat wrapping paper	82.6	70.2
Paper picnic plates	9.5	4.1
White cotton fabric	2.6	3.8

[1] Each value represents the average per cent recovery from triplicate determinations.

Studies in which *B. globigii* spores were employed to determine if the presence of organic soils on surfaces affected the recovery rate of the DSAP method showed that certain soils materially interfered with quantitative recovery. These date are outlined in Table 3 and show that less than 15% of the spores were recovered on surfaces containing the fatty and whole-egg soils, whereas 74 to 91% were recovered from surfaces overlaid with the water soluble materials.

TABLE 3

Recovery of Bacillus globigii spores from china surfaces contaminated with a variety of spore-soil mixtures

Soils	Number of bacteria per 10 ml. of uninoculated soil	Number of spores per 0.1 ml. of spore-soil mixture [1]	Number of spores recovered by the direct surface agar plate method [1]	Per cent recovery
Water	0	630	569	90.3
	0	380	347	91.3
Whole egg suspension	0	523	97	18.5
	0	273	21	7.7
Milk (10% solids)	0	630	557	88.4
	0	337	297	88.1
Hydrogenated vegetable shortening	0	633	110	17.4
	0	317	34	10.7
Sucrose (10% solution)	0	687	508	73.9
	0	323	263	81.4
Prepared cake mix (10% suspension)	0	703	526	74.8
	0	330	262	79.4

[1] Each value represents the average of triplicate determinations.

DISCUSSION

Results of experiments in which *B. globigii* spores were recovered from china plates by the direct surface agar plate method revealed that this technique is capable of detecting bacterial contamination on surfaces with a high degree of precision. It is interesting that, although the method closely approaches quantitative recovery with excellent precision it does not on the average recover all the spores inoculated on the surface.

The less than 10% difference in numbers of spores present in the inoculum and that recovered from the plates appears to result from deleterious changes that occur during drying on the surface. This is substantiated by the results of the micrococcal experiments, in which considerably less than quantitative recovery was obtained in comparison to the original inoculum. The vegetative cells are quite susceptible to the lethal effects of drying and this susceptibility resulted in an apparently poorer recovery rate. However, there is no reason to believe that this technique preferentially detects spores to vegetative cells; therefore, the poor recovery of micrococci can be attributed to a higher death rate during drying. In spite of the effect of drying, the method retained its high precision comparable to that of a plate count technique.

Though this method is presently limited to flat, nonporous surfaces and is probably too complicated for practical application routinely, it has the advantage over other recovery methods because *in situ* determinations are made, thus eliminating errors associated with intermediate manipulations.

The method presents possibilities in such areas as testing residual effects of disinfectants and sanitizers, determinations of the persistence of bacteria on surfaces under various ambient conditions, determining the bacteriostatic and fungistatic effects of paints and various coating materials, and as a laboratory procedure for the quantitative comparison of surface recovery methods in terms of the proportion of contamination recovered and precision of the results.

SUMMARY

A direct surface agar plate (DSAP) laboratory method for detecting the bacterial contamination on nonporous surfaces is described. Based on averages of triplicate determinations, the DSAP method is capable of detecting, in 95 trials out of 100, 88.5 to 99.3% of the spore contamination on nonporous surfaces. A much wider variation in results between experiments was observed in the micrococcal experiments due to differences in susceptibility to drying among the individual cultures employed. However, for any individual experiment the variation was not too extensive and the 95% tolerance limits were found to be 91.2 to 109.7% of the average per cent recovery for that experiment, 95 trials out of 100.

Comparison of the precision of this method with that of a conventional plate count technique showed that the variation between replicate counts of both methods was equivalent with either spores or vegetative cells.

The character of both the surface itself and the film of soil on the surface affect the recoveries attainable by the DSAP technique.

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LITERATURE CITED

1. A.P.H.A. *Standard Methods for the Examination of Dairy Products.* American Public Health Association, New York, N. Y. 10th ed., 1953.
2. A.P.H.A. *Thirteenth Year Book, 1947–48. Am. J. Public Health,* Part 2, **38**, 68 (1948).
3. A.P.H.A. *Fourteenth Year Book, 1948–49. Am. J. Public Health,* Part 2, **39**, 66 (1949).
4. Buchbinder, L., Buck, T. C., Jr., Phelps, P. M., Stone, R. V., and Tiedeman, W. D. Investigations of the swab rinse technic for examining eating and drinking utensils. *Am. J. Public Health,* **37**, 373–378 (1947).

A COMPARATIVE EVALUATION OF METHODS FOR DETERMINING THE BACTERIAL CONTAMINATION OF SURFACES [a]

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Numerous investigations have been conducted to fill the need for simple, reliable, bacteriological tests to determine quantitatively the sanitary quality of food contact surfaces. They have resulted in the development of various swabbing *(3, 4, 5, 7, 10)*, rinsing *(2, 11)*, and agar contact methods *(6, 8, 9, 12)*. Some methods are better suited for examining flat surfaces than for examining food containers, eating utensils, or processing equipment, etc.; in fact, there is no one method, presently available, that is applicable to the diversified surfaces encountered in processing plants, restaurants, and related food handling establishments. The plant sanitarian is therefore confronted with the problem of deciding which of the available methods will meet his requirements most satisfactorily. Moreover, information is not available on two important factors to be considered in making such a decision; namely, the average proportion of the total contamination that a particular method recovers and the reliability of its results.

Recently a direct surface agar plate laboratory method (DSAP) *(1)* has been developed which, based on triplicate determinations, is capable of detecting 88.5% to 99.3% of the bacterial spore contamination on nonporous surfaces in 95 trials out of 100. This 95% confidence interval is based on experiments in which triplicate plate counts of a contaminating suspension were used as the bases for these percentage recoveries. In addition, the coefficient of variation of individual DSAP counts was shown to be 7.1% which is equivalent to the precision of the conventional agar pour plate method of enumerating bacteria. Although this method is probably too complicated for practical application routinely, it has provided an excellent reference procedure for quantitative comparison of surface recovery techniques in terms of the proportion of contamination detected and the precision of the results. Employing the DSAP method as the reference procedure a comparison was made of representative recovery methods and the results evaluated statistically.

EXPERIMENTAL PROCEDURE

Inoculation of test surfaces

1. Micrococcus pyogenes var. *aureus* 209. Maintenance of stock cultures and preparation of milk suspensions were identical to the procedures previously described *(1)*. The final suspensions contained approximately 10,000 to 13,000 organisms per ml. as determined by triplicate plate counts on tryptone glucose extract agar incubated at 35° C. for 48 hours.

Vitreous china salad dishes were selected as test surfaces, because they are readily available, can be easily manipulated, and their smooth nonporous surfaces are typical of

[a] This work was supported in part by the Federal Civil Defense Administration under Delegation No. 1 to the U. S. Department of Health, Education, and Welfare.

those encountered in food handling equipment. The total area of the food contact surface was 254 sq. cm. Just prior to use, the dishes were detergent washed, water rinsed, steam sterilized and flame seared. After cooling, a 0.4 ml. aliquot of the milk suspension was pipetted onto the center of each dish. Immediately after dispensing the inoculum, each dish was brushed with an individual, dry, sterile typewriter brush[b] to distribute the organisms evenly over the whole of the plate surface. Preliminary studies revealed that a 0.4-ml. aliquot spread with this particular type of brush resulted in an even distribution of the contamination over the dish surface without running, beading, or pool formation. Dishes treated in this manner required exactly 8 minutes to become visibly dry at 60% RH and 80° F.

2. Bacillus globigii spores. Spore suspensions were prepared as previously described *(1)* and the china dishes were contaminated by spreading a 0.1-ml. aliquot of the final aqueous spore suspension over a 4.0-sq.-in. area in the center of a dish and permitting it to dry 10 minutes at ambient conditions. Temperature and relative humidity varied within the limits of 70 to 85° F. and 21% to 55% RH (sling psychrometer).

Methods for the examination of contaminated surfaces

1. APHA cotton swab (1 swab per 4 plates). The procedure recommended by the American Public Health Association for the bacteriological examination of food utensils *(3, 4)* was employed.

2. Cotton swab (1 swab per plate). The method deviated from the APHA procedure in that a single swab was used to swab one dish and was broken off into 10 ml. of the buffered rinse solution.

3. Calcium alginate swab (1 swab per plate). Machine rolled swabs were employed.[c] The method was similar to that for the cotton swab, except that ¼ strength Ringer's solution was used in place of the buffered rinse solution and 1.0 ml. of an aqueous 10% sodium hexametaphosphate solution was added to 9.0 ml. of the Ringer's solution just before shaking to dissolve the calcium alginate.

4. Agar syringe method. A modification of the method proposed by Litsky *(13)* was used. A 100-ml. hypodermic syringe, from which the end had been cut to obtain a hollow cylinder, was sterilized and filled with tryptone glucose extract agar. After hardening, a layer of medium was pushed beyond the end of the barrel by means of the plunger and pressed against the center of the contaminated dish surface for 5 seconds. After contact, the layer of medium was cut off with a sterile spatula, placed in a Petri dish, and incubated 24 hours at 35° C.

5. Surface rinse-agar plate count. Twenty ml. of buffered rinse solution *(2)* was pipetted onto the dish and the contaminated area was scrubbed (100 strokes) with a sterile rubber policeman. After scrubbing, 10 ml. of the rinse was distributed in three Petri dishes and tryptone glucose extract agar pour plates were prepared. The pour plates were incubated 48 hours at 35° C.

6. Surface rinse-membrane filter count. Following the rinse and scrubbing described immediately above, the total 20 ml. of rinse solution was added to a Millipore Filter funnel apparatus containing 100 ml. of sterile rinse solution and filtered through a membrane. This was followed by flushing the contaminated surface of the dish with an additional 100 ml. of sterile rinse solution and collecting the rinse directly in the funnel. As a final rinse, the funnel was flushed with 100 ml. of sterile rinse solution. The membrane was placed on a nutrient pad containing 1.8 ml. of double strength tryptone glucose extract broth and incubated 24 hours at 35° C.

7. Direct surface agar plate method (DSAP). The procedure of Angelotti and Foter *(1)* was followed. Tryptone glucose extract agar and incubation conditions of 35° C. for 18 hours were employed.

Modifications in the above procedures will be discussed in relation to the results of the experiments to which they apply.

RESULTS

Validation of experimental conditions. Previous investigation indicated that the effect of certain experimental conditions had to be determined before conducting a critical

[b] Obtained from Federal Prison Industries, Inc., U. S. Dept. Justice Stock Number 53B-26020, 100% bristles, 1 in. wide x 1¼ in. long x ¼ in. thick.

[c] Supplied by Splain and Lloyd, Inc., Milford, Ohio.

comparison of surface recovery methods. Thus, before using *M. pyogenes* var. *aureus* 209 as a surface contaminant it was necessary to determine: (a) the death rate of the organism when spread as an inoculum over the test surface, (b) the distribution of organisms over the surface, since the various methods sampled areas of dissimilar dimension, and (c) the variation in number of organisms on the replicate contaminated surfaces.

To determine the effects of these variables, duplicate experiments were performed employing 10 dishes per experiment (2 rows of 5 dishes) contaminated as described above. At exactly 8 minutes from the time the first pair of dishes was contaminated, the initial or zero time count was made by performing two DSAP determinations on the center and rim of each dish (area sampled by each DSAP determination was 3.8 sq. cm.). Similar pairs of inoculated dishes were sampled at intervals of 20, 40, 60, and 80 minutes after drying.

Table 1 shows the average values obtained from DSAP determinations made on the pairs of dishes in the two experiments. Observed counts were transformed to square roots to normalize the distributions of replicate values obtained from each dish and were then examined by an analysis of variance.

TABLE 1

Number and distribution of Micrococcus pyogenes var. aureus 209 recovered by DSAP method from glazed china surfaces exposed for different times to constant temperature and relative humidity conditions

Time lapse after initial 8-minute drying period (minutes)	Experiment I 58% RH and 80° F.		Experiment II 60% RH and 79° F.	
	No. from center of plate[1]	No. from rim of plate[1]	No. from center of plate[1]	No. from rim of plate[1]
0	43.9[2]	39.8[2]	48.3[2]	46.9[2]
20	52.9	46.2	49.0	50.8
40	40.0	39.4	28.4	48.3
60	34.8	48.0	34.5	53.3
80	29.4	32.8	36.3	42.9

[1] Average of 4 determinations, each consisting of duplicate direct surface agar plate determinations (area sampled 3.8 sq. cm.) from each of 2 plates.
[2] Square of average square roots.

Analysis showed that the results of single determinations made on the same location (rim or center) of replicate dishes did not vary more than the results of duplicate determinations from the same location of a single dish. In other words, there was no detectable difference in the numbers of organisms on replicate dishes.

Variance of the square roots of all the 0- and 20-minute direct surface agar plate determinations was found to be 0.224—actually less than the average "residual" variance (0.399) between duplicate samplings taken at the same time from the same location of a single dish. Thus, it was indicated that equal distribution of the organisms on the dish surface was obtained and that no variation due to death of the cells occurred on the dishes sampled at 0 and 20 minutes. However, variation due to death did occur after 60 minutes in the first experiment and after 40 minutes in the second. Death occurred more rapidly among organisms located in the center of the dish than among those located on the rims. This indicated that equal distribution of organisms could not be expected to persist on dishes which remained for more than 20 minutes at 80° F. and 60% relative humidity. Therefore, it was decided that all subsequent experiments employing *M. pyogenes* var. *aureus* 209 would be completed within 20 minutes.

Experimental design. Table 2 illustrates the experimental design employed in the comparative evaluation of methods for recovering *M. pyogenes* var. *aureus* 209. Eight sets of 15 dishes each were employed. Two sets of 15 dishes were contaminated on 4 consecutive days employing milk suspensions of the test organism. A fresh suspension of a 24-hour slant culture was prepared each day. Fifteen dishes were contaminated, dried, and sampled by the seven recovery methods employing the random order of testing shown in Table 2. Upon completing this procedure for one set of 15 dishes, the second set of 15 was similarly treated. Negative control dishes were incorporated into each of the experiments to determine the extent of possible airborne contamination and these were sampled by the DSAP method.

TABLE 2

Experimental design for a comparative evaluation of various surface recovery methods in recovering Micrococcus pyogenes var. aureus 209 from china plates

Surface recovery methods	No. of plates sampled per experiment	Random sequence of testing in 8 experiments			
		I and II	III and IV	V and VI	VII and VIII
Direct surface agar plate controls	4	6	2	8	2
APHA cotton swab (1 swab per 4 plates)	4	4	8	2	4
Cotton swab (1 swab per single plate)	1	5	4	3	8
Alginate swab (1 swab per single plate)	1	2	6	5	3
Agar syringe	1	8	3	4	7
Surface rinse-membrane filter rinse count	1	3	7	6	5
Surface rinse-agar plate rinse count	1	7	5	7	6

Direct Surface Agar Plate negative controls were employed in positions 1 and 9 for each experiment.

Recovery of organisms by test methods. Table 3 shows, for each method, the numbers of micrococci recovered per dish and the percentage recovery based upon the direct surface agar plate control count. For clarity in presentation, the random order in which the seven methods were tested is not shown but may be observed in Table 2. The negative controls are also omitted, since aerial contamination was insignificant.

A statistical analysis of the data obtained in these experiments employing micrococci as contaminants was conducted. The counts of the organisms recovered by each of the methods were first transformed to square roots in order to approximately normalize the distributions of replicate counts and to equalize errors in computed averages and in the ratios of these averages. Table 4 shows the results of the statistical analysis and gives, for each of the methods employed, the 95% confidence interval for the average per cent recovery, overall coefficient of variation (or percentage of error) and the percentage error ascribable to errors of teachnique alone. A method for separating technique error from the total variation in the observed counts is presented in a later section of this report.

Results in Table 4 show that, under the experimental conditions of known numbers or organisms per unit area of surface, equal distribution of the organisms over the test surface, and no variation in numbers of organisms due to death during the experimental time interval, the methods could be categorized into three general groupings. The APHA cotton swab method (1 swab per 4 surfaces) and both rinsing procedures yielded high recoveries but displayed rather wide variations among replicate determinations; the alginate and cotton swabs (1 swab per single surface) yielded low recoveries with very wide variations; and the agar syringe method yielded low recoveries but only slight variations between replicate determinations.

Comparison of results between the APHA cotton swab (1 swab per 4 surfaces) and the cotton swab (1 swab per single surface) methods revealed that significant differences in both total recovery and in the amount of variation between replicate determinations existed between these two methods. In swabbing surfaces upon which the distribution of organisms is unknown, the use of a single swab on 4 utensils has apparent advantages. When identically contaminated surfaces were swabbed, the APHA method recovered a greater percentage of the contamination with less variation than did the other method. No explanation has been found for these results and further investigation is warranted.

Table 5 shows the results obtained when the methods were employed to recover *B. globigii* spores. These studies were not conducted under the highly controlled conditions of time, temperature, and humidity existing in the experiments with micrococci. They also differed in that the inoculum was spread over a 4.0-sq.-in. area in the center of the dish by means of a sterile wire which resulted in uneven distribution of the organisms over the area. The procedure employed for the cotton and alginate swabs differed from

TABLE 3

Recovery of Micrococcus pyogenes var. aureus 209 from experimentally contaminated china plates by various methods

Experiment No.	Plate count: No. of org. per 0.4 ml. of milk suspension	DSAP control	APHA cotton swab (1 swab, 4 plates)		Cotton swab (1 swab, 1 plate)		Alignate swab (1 swab, 1 plate)		Agar syringe		Surface rinse agar plate count		Surface rinse MF rinse count	
			No. rec.[1]	% of control	No. rec.[2]	% of control	No. rec.[2]	% of control	No. rec.[3]	% of control	No. rec.[4]	% of control	No. rec.[4]	% of control
I	4,080	2,926	2,241	76.6	1,793	61.3	1,307	44.7	1,440	49.2	2,100	71.8	2,180	74.5
II	4,080	2,885	2,615	90.6	878	30.4	1,158	40.1	1,388	48.1	2,440	84.6	2,600	90.1
III	4,000	2,804	1,457	52.0	1,961	69.9	1,233	44.0	1,309	46.7	2,900	103.4	2,800	99.9
IV	4,040	3,139	2,540	80.9	1,438	45.8	1,345	42.8	1,257	40.0	2,180	69.4	2,560	81.6
V	3,880	2,865	2,316	80.8	1,550	54.1	1,233	43.0	1,414	49.4	3,260	113.8	3,260	113.8
VI	4,240	2,865	2,353	82.1	1,251	43.7	710	24.8	1,466	51.2	2,760	96.3	1,840	64.2
VII	4,160	3,475	2,092	60.2	1,457	41.9	1,494	43.0	1,414	40.7	3,120	89.8	2,660	76.5
VIII	4,240	3,160	1,718	54.4	1,718	54.4	1,905	60.3	1,571	49.7	3,020	95.6	2,360	74.7

Values represent the number of organisms recoverable from the whole plate surface and were derived by multiplying the actual number of organisms counted by the area factor and reciprocal of the dilution.

[1] Number of organisms counted $\times \frac{254 \text{ sq. cm. (total area of plate)}}{68 \text{ sq. cm. (area of plate swabbed)}} \times 10.$

[2] Number of organisms counted $\times \frac{254 \text{ sq. cm. (total area of plate)}}{68 \text{ sq. cm. (area of plate swabbed)}} \times 5.$

[3] Number of organisms counted $\times \frac{254 \text{ sq. cm. (total plate area)}}{9.7 \text{ sq. cm. (area plate sampled)}}$ (No dilution factor involved.)

[4] Number of organisms counted $\times \frac{254 \text{ sq. cm. (total plate area)}}{254 \text{ sq. cm. (area plate sampled)}} \times 20.$

TABLE 4

Statistical comparison of six methods for recovering Micrococcus pyogenes var. aureus 209 from experimentally contaminated china plates

Type of recovery method	$\bar{A}$	k	$\bar{X}$	f (per cent)	95% C. I. (per cent)	C. V. (x) (per cent)	s_e (per cent)
Control (direct surface agar plate)	296	10.16	3010	100	.....	8	5
High recovery, average precision							
(1) Rinse-plate count	135	20.00	2710	89.9	78–103	16	14
(2) Rinse-MF count	126	20.00	2520	83.6	71– 97	17	14
(3) APHA cotton swab (1 per 4 plates)	58	37.40	2150	71.5	60– 85	18	13
Low recovery, poor precision							
(1) Cotton swab (1 plate)	80	18.70	1490	49.5	40– 61	23	19
(2) Alginate swab (1 plate)	68	18.70	1280	42.5	35– 51	26	23
Low recovery, excellent precision							
(1) Agar syringe	54	26.19	1410	46.7	43– 50	7	0

$\bar{A}$ = average no. of organisms actually counted.

$k = \text{multiplication factor} = \frac{\text{area of plate sampled}}{\text{total area of plate}} \cdot 100.$

$\bar{X} = k \cdot \bar{A} = \text{average estimate of the number of organisms on the whole plate.}$

$f = \%\ \text{recovery} = \frac{\bar{X}\ \text{test method}}{\bar{X}\ \text{controls}} \cdot 100.$

95% C. I. = confidence interval for average percentage recovery from an infinite number of experiments.

C. V. (x) = coefficient of variation of x

$= \frac{\text{Std. dev. of } x \cdot 100}{\bar{X}}$ where x is an individual estimate of the total number of organisms per plate.

s_e = percentage error ascribable to technique errors alone. s_e does not include the portion of the variation in x which is due to Poisson variation in the numbers of organisms on the dried, replicate plates.

those previously described to the extent that the swabs were rubbed 25 strokes over the entire 4.0-sq.-in. contaminated area followed by an additional 25 strokes at right angles to the first. Plate counts of the 10 ml. of diluent were made by distributing it among three Petri dishes and preparing tryptose agar pour plates which were incubated at 35° C. for 48 hours. The remaining methods were applied in similar manner to those described for the micrococci, but tryptose agar was employed in the agar syringe and surface rinse-agar plate count methods, and a specially prepared broth medium[4] was used for the surface rinse-membrane filter count method.

The fourth column of Table 5 lists average percentage recoveries by each of the methods when the values obtained by the DSAP technique were taken as 100%. It is interesting to note that all the methods recovered a smaller proportion of spores than was observed with the vegetative cells. Subsequent study revealed that the exceptionally low recoveries obtained with the membrane filter method were due to an inability of the spores to colonize on the membrane rather than to any difference in actual removal by the scrubbing and rinse.

[4] Composition of broth: Proteose peptone, 30.0 g.; Glucose, 6.0 g.; l-alanine, 0.3 g.; Tween 80, 0.05%; Phosphate buffer 0.067M, pH 7.3, 1000 ml.

TABLE 5

Statistical comparison of six methods for recovering Bacillus globigii spores from experimentally contaminated china surfaces

Experiment number	Recovery methods	No. org. recovered from 4.0 sq. in by DSAP control (Av. of 3 replicates)	No. org. recovered from 4.0 sq. in. by recovery methods (Av. of 12 replicates)	Per cent recovery by test methods compared to DSAP	95% C. I.[1]	C. V. (x)[2] (per cent)	s_c[3] (per cent)
I	Cotton swab	99	36	36.4	30.1–43.2	25.4	19.0
II	Alginate swab	101	20	19.8	11.8–25.5	60.6	54.8
III	Agar syringe	112	48	42.9	33.8–50.9	28.5	15.3
IV	Surface rinse agar plate count	111	90	81.9	71.2–90.9	15.9	5.6
V	Surface rinse membrane filter count	110	21	19.1	15.5–21.7	22.8	5.7

[1] 95% confidence interval for average per cent recovery from an infinite number of determinations.
[2] Coefficient of variation of single determinations.
[3] Percentage error ascribable to technique error alone. s_c does not include the portion of the variation in x which is due to Poisson variation in the numbers of spores on the dried, replicate plates.

Although the average per cent recovery by the agar syringe method was approximately equivalent for both vegetative cells and spores, the extent of the variation between replicate determinations was significantly greater when spores were used. The wide variation observed in recovering spores reflects the influence of the experimental conditions on the results. Results observed with the micrococci revealed that, in spite of the relatively small proportion of the total surface area sampled by the agar syringe, the method was intrinsically precise when used to examine surfaces upon which the contamination was evenly distributed. However, results of the spore studies indicated that the small proportion of the contaminated surface area sampled combined with variation in numbers and distribution of the spores on the replicate surfaces may have contributed to a rather large error. Had a number of sites been examined on each of the replicate surfaces, a more reliable estimate of the spore contamination may have been possible, because of a reduction in error due to uneven distribution.

Though the precision of the cotton swab method was similar in the experiments employing both micrococci and spores, lower average recovery rates were observed with the latter. The fact that the whole 4.0-sq.-in. area of surface was swabbed and the complete 10 ml. of diluent was plated, reduces the significance of any difference in recovery attributable to sampling error. However, it was not possible from these experiments to determine the extent to which differences between spores and vegetative cells, in physical adsorption to the surface, manipulation of the swab, plating technique, or media contributed to the observed results.

In view of the rather close agreement in results by the alginate and cotton swabs (1 swab per dish) in recovering micrococci, the difference observed between the 2 types of swabs in the spore studies indicated that an inhibition of germination or growth may possibly have occurred. Investigations are presently under way to determine whether calcium alginate or sodium hexametaphosphate exhibit any inhibitory characteristics.

Good agreement in results was obtained with the surface rinse-agar plate count method in recovering both spores and vegetative cells. This agreement is probably attributable to the fact that the whole contaminated surface was scrubbed and rinsed, thus eliminating error caused by uneven distribution of the spore contamination over the surface.

Statistical method for estimation of technique errors. The precision of each of the methods, as measured by the variance between replicate determinations, was of as great interest as the total percentage recovery. Several sources of error might contribute to the

variation between replicate counts with the fundamental component arising from a Poisson distribution of organisms on the surface.

Variations in swabbing, pipetting, and related techniques may comprise an additional source of error. To test this possibility with respect to each of the 7 methods, the variance of each set of 8 replicate counts was divided by the corresponding mean count. In the absence of any source of error other than Poisson variation, these 7 ratios would be expected to average about unity. The observed average ratio was 2.80. One method, the agar syringe, showed a ratio of only 0.25; the others ranged from 1.69 to 4.62, indicating for these methods a variability significantly greater than that arising from Poisson variation alone.

To isolate and study these additional technique errors, each mean count (a measure of the Poisson component of variance) was subtracted from the variance, and the residuals plotted against the means. It appeared, in general, that technique errors in terms of numbers of organisms were proportional to the concentration under assay. One exception was the control (direct surface agar plate count) in which the mean count was higher but the variance less than in 4 of the 6 test methods. This control result strengthens the hypothesis of proportional test-method technique errors, since the control method alone did not involve such sub-sampling operations as scrubbing and pipetting.

Assuming, then, that the component of variance due to technique errors is proportional to the number of organisms put down on the surface, an algebraic method of estimating this component may be presented.

Let x denote, for a given method, the corrected estimate of the total surface count per plate and s denote the actual number of organisms put down on the surface and detectable by the method. We assume that s is Poisson distributed with mean km and variance k^2m. Then, on the basis of the above results, we may write:

$x = s + cs = s\ (1 + c)$, where c represents the proportional technique error with mean zero and variance σ_c^2. This model implies the following theoretical variance of x:

$$\begin{aligned}\text{Variance } (x) &= \text{Variance } (s) \cdot \text{Variance } (1 + c) + (km)^2 \cdot \text{Variance } (1 + c) \\ &\quad + (1 + 0)^2 \cdot \text{Variance } (s) \\ &= k^2m\,\sigma_c^2 + k^2m^2\,\sigma_c^2 + k^2m\end{aligned}$$

Now, x itself is the product of k, a combined area and volumetric correction factor, and A, the observed (uncorrected) surface count, i.e., $x = kA$. Therefore, Variance (x) is given empirically by the expression,

$$\sigma_x^2 = k^2 \text{ Variance } (A).$$

Equating this expression with the theoretical variance given above, we have:

$$k^2 \text{ Variance } (A) = k^2m\,\sigma_c^2 + k^2m^2\,\sigma_c^2 + k^2m$$

From this equation, solving for the variance of the proportional technique errors, we obtain:

$$\sigma_c^2 = \frac{\text{Variance } (A) - m}{m(m+1)}$$

Estimates, s_e, of the standard deviation for each method are presented in the last column of Tables 4 and 5. These estimates are computed by using $\overline{A}$, the sample mean, for m, the true mean, and s_A^2, the observed variance of the sample, for Variance (A), the population variance of all possible samples. That is,

$$s_e = \sqrt{\frac{S_A^2 - \overline{A}}{\overline{A}\,(\overline{A}+1)}}$$

By means of this procedure, it was possible to ascertain the technique error for each of the methods as an index of its reliability. By observing the percentage error ascribable to technique error alone, it is seen that the use of methods involving numerous manipulations which contribute to the total variance, such as those in the cotton swab technique, results in the manifestation of large technique errors and consequently yields unreliable estimates of the bacterial contamination present. Conversely, methods similar to the DSAP or agar syringe methods which make direct determinations of the surface con-

tamination and are unencumbered by intermediate manipulations, contain very little inherent technique error and yield precise results.

DISCUSSION

Results of the above experiments permitted an appraisal of several factors which require consideration when interpreting results obtained with some of the commonly employed surface recovery methods.

When uniformly contaminated surfaces were sampled in this study by the cotton swab technique, swabbing a number of similar sites on replicate test surfaces yielded both higher recovery and more precise determinations than did swabbing a single site with a single swab.

In field applications of the swabbing technique, where the variables of type and distribution of organisms also play a significant part in determining the results obtained, an additional advantage is gained by swabbing large areas. Samples obtained by swabbing large areas obviously will "average out" these distribution variations better than more localized samples obtained by swabbing smaller areas.

Therefore, the use of a procedure similar to that recommended by the American Public Health Association, in which a number of sites of fairly large area are sampled with a single swab, appears to be a more reliable method of determining the average bacterial contamination present than the use of a single swab for sampling one site of small dimension. In addition, the fact that swabbing procedures were shown to be subject to inherent errors which are directly attributable to the techniques themselves emphasizes the necessity for adherence to standardized procedures. The angle and pressure applied to the swab, the inability to swab exactly equivalent areas on replicate sites, the incomplete release of bacteria during shaking in diluent, and errors associated with pipetting and counting all contribute to the total variance of the results.

The nature of the cotton swab method is such that it is readily adaptable to sampling a variety of surfaces; because of this, it is used routinely for recovery work in spite of its limitations. Because results obtained with this method are affected by inherent technique error of rather large magnitude, and by the type and distribution of organism present, a clear cut interpretation of the results is often difficult to make. Consequently, an awareness of the various factors influencing the results, particularly the wide variations observed in replicate determinations, should be carefully considered when reporting results obtained by a swabbing procedure.

The agar syringe method is not applicable to as wide a variety of situations as the cotton swab. However, for flat or slightly rounded nonporous surfaces which are overlaid with a fairly continuous contamination, it apparently serves to better advantage than a swabbing technique. The results cited above indicate that the agar syringe so consistently recovered approximately half of the micrococcal contamination from the test surfaces, that had the results been multiplied by a factor of 2, a more reliable estimate of the total contamination would have been achieved than was possible by any of the other techniques. Though it is indicated that the method suffered a loss of precision when used to examine surfaces having a discontinuous spore contamination; its simplicity makes it a highly desirable field test, and it warrants consideration over the cotton swab in situations where routine, replicate determinations are required.

Rinsing techniques, particularly when accompanied by scrubbing, have a

distinct advantage over other recovery methods because the entire surface is sampled. Sampling the entire surface area reduces error introduced by inadvertent sampling of surface sites which are not contaminated in a manner typical of the remainder of the surface. Moreover, rinsing yielded higher recoveries than any of the swabbing procedures tested and had precision equivalent to the APHA cotton swab method and better than the alternate alginate or cotton swab procedures (1 swab per surface). Unfortunately, rinsing is limited in application, but for examining food containers or similarly suitable utensils, rinsing with concomitant scrubbing appears to be the method of choice.

SUMMARY

A comparative evaluation of six methods for recovering *Micrococcus pyogenes* var. *aureus* 209 and *Bacillus globigii* spores from china surfaces was conducted to ascertain the proportion of the contamination detected and the precision of the results obtained with each.

Results indicated that, under the experimental conditions employed, the methods could be categorized into the following groups based on their recovery of the micrococcal contamination: (1) the surface rinse-agar plate count, surface rinse-membrane filter count, and the APHA cotton swab (1 swab per 4 surfaces) methods yielded high average per cent recoveries with fairly good precision, (2) the cotton and alginate swab methods (1 swab per single surface) gave low average per cent recoveries and displayed very poor precision, and (3) the agar syringe method which yielded low average per cent recoveries, but excellent precision.

The results from the experiments employing *Bacillus globigii* spores revealed that all of the methods recovered a smaller proportion of the contamination than was observed with vegetative cells; and that the variables of type, distribution, number of organisms present, as well as the manner in which the procedures are applied significantly affect the results obtained.

The relationships of the above variables to practical field applications are discussed.

LITERATURE CITED

1. Angelotti, R., and Foter, M. J. A direct surface agar plate laboratory method for quantitatively detecting bacterial contamination on nonporous surfaces. *Food Research*, **23**, 170 (1958).
2. A.P.H.A. *Standard Methods for the Examination of Dairy Products.* 10th ed., 1953. American Public Health Association, New York, N. Y.
3. A.P.H.A. *Thirteenth Year Book, 1947–48. Am. J. Pub. Health*, Part 2, **38**, 68 (1948).
4. A.P.H.A. *Fourteenth Year Book, 1948–49. Am. J. Pub. Health*, Part 2, **39**, 66 (1949).
5. Barnes, J. M. The removal of bacteria from glass surfaces with calcium alginate, gauze and absorbent cotton wool swabs. *Proc. Soc. Appl. Bacteriol.* (Gr. Brit.), **15**, 34 (1952).
6. Barton, R. R., Gorfien, H., and Carlo, R. M. Determination of bacterial numbers on tableware by means of direct plating. *Appl. Microbiol.*, **2**, 264 (1954).
7. Cain, R. M., and Steele, H. The use of calcium alginate soluble wool for the examination of cleansed eating utensils. *Can. J. Public Health*, **44**, 464 (1953).
8. Flett, L. H., Guiteras, A. F., and Shapiro, R. L. A quantitative method for determining the bacterial contamination of dishes. *Appl. Microbiol.*, **2**, 100 (1954).
9. Hammer, B. W., and Olson, H. C. Bacteriology of butter. III. A method for studying the contamination from churns. Iowa Agric. Expt. Sta. *Bull. 141*, Ames, Iowa (1931).

10. HIGGINS, M. A comparison of the recovery of organisms from cottonwool, and calcium alginate wool swabs. *Month. Bull. Ministry Health and Pub. Health Lab. Service*, **19**, 50 (1950).
11. STEDMAN, R. L., KRAVITZ, E., AND BELL, H. Studies on the efficiencies of disinfectants for use on inanimate objects. I. Relative activities on a stainless steel surface using a new performance test method. *Appl. Microbiol.*, **2**, 119 (1954).
12. WALTER, W. G., AND HUCKER, G. J. Proposed method for the bacteriological examination of flat surfaces. *Am. J. Pub. Health*, **31**, 487 (1941).
13. WALTER, W. G. Symposium on methods for determining bacterial contamination on surfaces. *Bacteriol. Rev.*, **19**, 284 (1955).

INCIDENCE AND NATURE OF ENTEROCOCCI ON PLANT MATERIALS[a]

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During recent years, streptococci of the group known as enterococci or fecal streptococci have been isolated almost at will from frozen foods. Thus, Ferraro and Appleman *(8)* and Kaplan and Appleman *(15)* obtained them from frozen concentrated orange juice produced in several processing plants. Hucker, Brooks, and Emery *(12)* isolated predominantly gelatin liquefying strains from frozen peas, beans, and corn. Burton *(5)* isolated enterococci from 81% of post-blanched line samples, and from 89% of frozen vegetables and cantaloupe held for a year in storage at −20° C. Larkin, Litsky, and Fuller *(17)* recovered them from a wide variety of frozen fruits and vegetables, finding them absent only from the very highly acidic citrus juices.

Historically, the enterococci have been defined as a group of intestinal inhabitants by Dibble *(7)*, Bagger *(2)* and Alston *(1)*. The impression that these organisms reside only in the intestinal tract of certain warm blooded animals and of man has been fostered by the reports of Winter and Sandholzer *(29)* and Ostrolenk and Hunter *(24)*; and by the quite legitimate attempts to employ the presence of the enterococci in lieu of, or in addition to, the presence of the Gram negative, lactose fermenting bacteria as indices of pollution [France and Fuller *(9)*, Hajna and Perry *(11)*, Leininger and McCleskey *(18)*, Litsky, Rosenbaum, and France *(19)*, Mallmann *(20)*, Mallman and Litsky *(21)*, and Mallman and Seligmann *(22)*].

In view of the fact that relatively few studies have been reported concerning the distribution of the enterococci among other than animal sources, it is unfortunate that the stigma of human pollution is attached to, or implied by, their occurrence in frozen foods. The point has been raised by Kaplan and Appleman *(15)* and by Vaughn, Murdock and Brokaw *(28)*. Ostrolenk and Hunter *(24)* cultured only 2 samples of soil, from which enterococci were not recovered, and Medrek and Litsky *(23)* noted an incidence of only 2.2% in the examination of 369 samples of soils taken from the environment of reservoirs. Sherman's statement *(26)* to the effect that enterococci of the *Streptococcus faecalis* and *S. liquefaciens* types occurred rather commonly on plants appears to have been overlooked until recently.

Studies reported in this paper were undertaken to determine to what extent the enterococci may occur apart from the intimate human and animal environment, and to determine whether the presence of the organisms in frozen foods could be ascribed to sources other than those arising through human negligence. Because of the recent finding that the species known as *S. faecalis*

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may consist of two closely related species, *S. faecalis* and *S. faecium*, and that the latter may be of other than human origin [Cooper and Ramadan *(6)*, Barnes *(3)*, Barnes and Ingram *(4)*, Ingram and Barnes *(14)*, and Skadhauge *(27)*], isolates were studied for species identification. At the same time, most samples were submitted to cultural analysis for the presumptive presence and limited identification of the Gram negative, lactose fermenting, coliform bacteria.

EXPERIMENTAL

Samples of soils were taken from tilled and inhabited areeas and also from such areas as forest and waste lands to which human access is limited. Source material of plants, including leaves, flowers and shoots were severed with instruments sterilized in alcohol and gathered in sterile containers. The majority of the samples were taken from plants grown in open suburban and rural fields, grassland, farms and orchards, selected where possible from sites and plant elevations at which contact with domestic and wild animals and man is quite improbable. Materials were not taken from areas such as playgrounds, lawns, and grazing fields where probability of contamination appeared obvious. Several lots of vegetables were taken from agricultural plots which received only mineral fertilizer and which were worked only with machinery. Ovules of beans were removed after the outer curve of the pod had been slit. The aril of the ovule was ruptured as it was introduced into the culture medium. Ovules of corn were removed from the cob after a window had been cut through the husk. From one to 10 ovules were cultured in each tube of broth, so that the 27 trials reported in Table 2 represent approximately 125 ovules. Plates of azide dextrose agar were exposed in a freezing processing plant packing green beans at the dumping area, over the outlet of the blanchers, over the sorting belts, over the French bean line, and over trays of the product before these entered the freezing tunnel.

Except for ovules, plant materials were disintegrated with a liquidizer in 9 volumes of water. Dilutions containing 0.1, 0.01 and 0.001 g. of material were introduced into 3 tubes of azide dextrose broth at each dilution. From tubes with growth upon incubation at 37° C., transfers were made successively to broth incubated at 45° C., and then to agar plates to obtain isolates.

Streptococci which grew at 10° C. and at 45° C. were retained for further study. All those retained grew at pH 9.6 in dextrose broth, and most isolates also grew in broth containing 6% NaCl and reduced 0.1% methylene blue in milk.

Ultimate species identification was based upon digestion of gelatin and casein, hemolysis of sheep red blood cells, fermentation of glycerol, alpha-methyl-glucoside, and citrate [Lake, Deibel, and Niven *(16)*] arabinose and sorbitol, and growth upon dextrose agar containing 0.04% potassium tellurite or 0.3 unit of penicillin per ml.

RESULTS

Table 1 presents the results of a preliminary survey to ascertain the approximate distribution of streptococci in soil and on plants of cultivated areas, as contrasted with the occurrence in soil and on plants of wastelands and limited access areas owned by the Tennessee Valley Authority. The organisms were recovered from 62% of the soil

TABLE 1

The isolation of confirmed streptococci from sources in cultivated and in uninhabited areas

Source	Cultivated areas		Uninhabited areas	
	Number of samples	Number containing streptococci	Number of samples	Number containing streptococci
Soil	14	8	10	1
Plants	20	13	21	6
Totals	34	21	31	7
Per cent		62		22

TABLE 2

The occurrence of enterococci and of Gram negative, lactose fermenting bacteria on flowers, leaves, and grasses of plants

Streptococci and coliform bacteria [1]	Streptococci and Escherichia spp.	Streptococci only	Coliform bacteria only [1]	Escherichia spp. only	Neither obtained
Flowers:					
Angelica (2)[2]		Chrysanthemum (2)	Bachelor button	Burdock	Nettle, horse
Cherry, wild		Clover, hop	Burdock (2)	Rose, cult.	Privet (2)
Chrysanthemum		Cherry, wild	Clover, red		Yarrow
Clovers (4; red, white, dutch)		Daisy, s. e.	Daisy fennel		
Daisy, s. e.		Petunia[3]	Honesysuckle		
Honeysuckle (4)		Senecio	Milkweed (2)		
Lilly, day			Privet		
Rose, cult. (4)			Rose, cult.		
Yarrow			Yarrow		
Leaves:					
Asparagus	Buckthorn	Blackberry	Asparagus (2)	Box elder	Begonia[3]
Bean, cult.	Columbine	Black walnut	Box elder	Peach	Cherry, cult.
Blackberry	Lettuce, cult.	Plantain, b. l.	Lettuce, wild		Coleus[3]
Cedar, white	Mint	Poison ivy	Loco weed		Corn
Columbine	Poke weed (2)		Peach		Euonimus[3]
Elm			Staghorn		Geranium[3]
Mulberry					Ivy[3]
Lettuce, wild					Lilac
Plantain, n. l.					Maple (2)
Ragweed					Oleander[3]
					Onion
Grasses:					Sassafras (2)
Corn tassel	Johnson Grass (2)	Orchard	Corn		Snapdragon (2)[3]
Fescue	Oats, wild (2)	Rye	Johnson grass		
Orchard (4)			Oats, wild		
Rye					

[1] Gram negative, lactose fermenting rods other than *Escherichia* spp.
[2] Figure in parentheses indicates number of trials with the source.
[3] Plants grown in greenhouse.

and plant samples taken from farm lands and yards, and from 22% of the samples from non-agricultural areas.

Results of the survey of plants for the occurrence of streptococci and the Gram negative, lactose fermenting bacteria are presented in Table 2. A distinction is made between those coliform bacteria which were identified tentatively as being species of the genus *Escherichia* and others. Many of the latter appeared to be *Aerobacter aerogenes*.

Enterococci were isolated in 62 of 106 trials. Their occurrence with *Escherichia* spp., with other coliform bacteria, and with none of these is listed in the first 3 columns of the table. Some items are listed in more than one column. Thus, enterococci were present with and without coliform bacteria on flowers of wild cherry and small-eyed daisy, on blackberry leaves, and on orchard and rye grasses.

Duplication of entries may also be noted in the 2 columns which list sources of coliform bacteria from which streptococci were not obtained. These are flowers of the cultivated rose and of burdock, and leaves of box elder. The final column lists a total of 20 trials during which neither type of organism was recovered. These sources include 7 plants sampled in the greenhouse. Flowers of petunia, also taken from the greenhouse, contained enterococci. Flowers of wild privet and of yarrow, and the leaves of corn, were free of both types of organisms in some instances, and contained coliform bacteria or streptococci or both in others.

As shown by Table 3, enterococci are present on green beans and on the outer leaves of cabbage, and in very small numbers of ovules of beans and of corn. Recoveries from

TABLE 3

The isolation of confirmed enterococci from economic, edible plants

Plant	Number of trials	Number of times enterococci were recovered
Beans, green	44	27
ovules	325	9
Broccoli, leaves	5	0
flowerets	20	0
Cabbage, outer leaves (after kitchen trim)	9	6
inner leaves	3	0
Cauliflower flowers	25	0
Corn ovules	27	16
Strawberries	30	0

green beans appear low, because during part of this study organisms which failed to meet one or more of the Sherman criteria were discarded. Streptococci were not recovered from leaves and flowerets of broccoli nor from the flowers of cauliflower, from the inner leaves of cabbage, or from strawberries. Six of 7 samples of green beans taken from a field worked only by machinery contained streptococci. The broccoli and 10 of the heads of cauliflower were taken directly from a large field which had been given overhead irrigation with river water. The remaining cauliflower, trimmed of leaves at the site of harvest and subjected to intimate contact with the hands, was sampled at the processing plant.

Identity of the streptococci. Of 142 isolates, 136 were typical enterococci which grew at 10° C. and at 45° C., in broth containing 6.5% NaCl, in broth adjusted to pH 9.6, and which reduced methylene blue in milk. All except one produced a greening reaction on agar containing sheep's blood.

Two subgroups were distinguished. Those which are designated as *S. faecalis*-like grew readily on agar containing potassium tellurite, reduced tetrazolium strongly, and fermented glycerol anaerobically and citrate. None, however, fermented alpha-methyl-glucoside. The majority of these cultures hydrolyzed casein.

Cultures termed *S. faecium*-like failed to grow on tellurite agar, failed to ferment glycerol anaerobically, and did not ferment melizitose, but did ferment mellibiose. As summarized in Table 4, the isolates were variable with respect to final pH in dextrose broth, reduction of tetrazolium, growth on agar containing penicillin, in the fermentations of arabinose and sorbitol, in the ability to decarboxylate tyrosine and to produce the

TABLE 4

Physiologic reactions of the streptococci isolated from plant materials

Reaction	*S. faecalis*-like	*S. faecium*-like
Final pH in dextrose	3.8–4.5	66–3.8–4.5 28–4.6–4.9
Reduction of tetrazolium:		
strong	40	1
weak	2	38
none	1	52
Growth on 0.3 unit penicillin:		
good	35	29
scant		14
none		16
Fermentations:		
arabinose	0	46
sorbitol	44	0
both sugars	2	21
neither sugar	0	27
Decarboxylase activity:		
0.8–2.2 ml. N/10 HCl	30	15
0.2–0.6 ml. N/10 HCl	7	55
No decarboxylation	0	7
Ribonuclease activity:		
good	5	10
moderate	1	10
none	2	22

enzyme ribonuclease. Very little correlation occurs among the cultures. Ninety-four cultures were placed into this group.

The distribution of the species of streptococci according to origin is shown in Table 5. *S. faecalis*-like organisms were isolated from a small number of plants in the general survey, from green beans in the field and during processing after blanching, from the atmosphere of the processing plant, and from ovules of corn. Although not encountered in the ovule of the bean, too few cultures were obtained from this source to warrant a conclusion. Casein liquefying variants were isolated from all sources except from the beans; this is in contrast to the findings of Hucker *et al. (12)*. The majority of the streptococci in all categories were those identified as being similar to *S. faecium*.

DISCUSSION

Three facts emerge from this study: (1) that streptococci physiologically falling into the taxonomic group known as the fecal streptococcus or the enterococcus do occur commonly on plant surfaces in an agricultural and an inhabited environment, and in soils under cultivation or in the vicinity of cultivation; (2) that the majority of the streptococci isolated from plants in

TABLE 5

Identification of enterocci according to origin

Source	*S. faecalis*-like	*S. liquefaciens*-like	*S. faecium*-like
Miscellaneous plants	3	20	56
Field beans	3	0	7
Beans in process	3	0	5
Plates exposed in processing plant	3	2	30
Corn ovules	4	3	5
Bean ovules	0	1	2

this study appear to be physiologically different from any species described to date, conforming most closely to that known as *S. faecium;* and (3) that they may enter a food item to be frozen via the atmosphere. The latter fact was obtained during the freezing processing of green beans, and application to other fruits and vegetables which are frozen should be made with reservation, pending further study.

At the moment, nothing is known as to the origin of the streptococci which have been isolated from plants. It is possible that they originate with animal sources; on the other hand, it is also possible that they occur on plants in an epiphytic relationship. The rather consistent association with green beans, and the failure to isolate them from the many samples of broccoli, cauliflower, and strawberries, is compatible with our knowledge of bacterial epiphytism on plants. Isolation in some trials but not in others with some plants, however, also indicates a random contamination by one or more distributive agencies.

If the enterococci were to originate primarily from warm blooded animals, it would seem likely that many isolates of *E. coli* would have been obtained. It has been shown that the fecal coliform bacteria may persist in soil for an appreciable period of time *(21)*. Haenel and Mueller-Buethow *(10)* concluded that enterococci and *E. coli* either will occur together in feces of animals, or else both will be absent. Studies by Randall *(25)* seem to substantiate this observation, for he obtained *E. coli* from 6 of 8 samples of soils which contained enterococci. It is unfortunate that Randall did not study his isolates of the enterococci in more detail. During the current study, known Gram negative intestinal residents were isolated only infrequently.

With this background, one may question the significance which may be attached to the presence of the enterococci in frozen foods at the present time. Even without the knowledge of a possibly new botanical strain of streptococcus, Vaughn *et al. (28)* see little cause for alarm over the presence of streptococci in frozen citrus juices, although the origin has not been clearly established. Hunter *(13)* has stressed the necessity for "sufficient study of the methods of production and handling of the class of food product under control" for the proper interpretation of the significance of members of the coliform group, and this statement could well apply to the enterococci as well. With the demonstrated route of entry of these organisms onto processing lines and into frozen foods via the atmosphere, one may suggest that the question of significance should be held in abeyance, pending further studies of the ubiquity of these organisms in nature, their origins, their identity, and alternative routes of entry during processing.

SUMMARY AND CONCLUSIONS

Studies were undertaken to determine the frequency with which organisms of the group known as enterococci occur in nature, their identity, and the possibility of a route alternative to pollution into frozen foods.

In a comparative study, the enterococci were isolated from 62% of samples of plants and soils taken from agricultural and inhibited areas, and from 22% of similar samples taken from unpopulated areas reasonably devoid of human and large mammalian life. They were isolated from 58.5% of 106 samples involving 63 plant species, with frequency from green beans, and from a small per cent of the ovules of corn and of green beans. They were also isolated

from the atmosphere of a freezing processing plant. They were not isolated from a total of 50 samples of broccoli or of cauliflower, nor from 30 samples of strawberries.

In 46 instances, coliform bacteria were associated with the streptococci. Few of the coliform bacteria were of the genus *Escherichia*. In 13 instances, streptococci occurred without the coliform bacteria, in 21 instances coliform bacteria were isolated without obtaining streptococci, and in 20 instances neither type of organism was obtained.

A small per cent of all isolates studied were similar to *S. faecalis*, and a somewhat larger per cent were a variant capable of digesting casein. Most isolates appear to be similar to but not identical with *S. faecium*.

Although there is no knowledge regarding the origin or the natural habitat of the latter type of streptococcus, the failure to isolate other known intestinal residents concurrently indicates that it is not of human or of warm blooded animal origin.

LITERATURE CITED

1. ALSTON, J. M. An investigation of streptococci isolated from the alimentary tract of man and certain animals. *J. Bacteriol.*, **16**, 397 (1928).
2. BAGGER, S. V. The enterococci. *J. Pathol. Bacteriol.*, **29**, 225 (1926).
3. BARNES, E. M. Tetrazolium reduction as a means of differentiating *Streptococcus fecalis* from *Streptococcus faecium*. *J. Gen. Microbiol.*, **14**, 57 (1956).
4. BARNES, E. M., AND INGRAM, M. The identity and origin of fecal streptococci in canned hams. *Ann., Inst. Pasteur de Lille*, **7**, 116 (1955).
5. BURTON, M. O. Comparison of coliform and enterococcus organisms as indices of pollution in frozen foods. *Food Research*, **14**, 434 (1949).
6. COOPER, K. E., AND RAMADAN, F. M. Studies in the differentiation between human and animal pollution by means of faecal streptococci. *J. Gen. Microbiol.*, **12**, 180 (1955).
7. DIBBLE, J. H. The enterococci and fecal streptococci. *J. Pathol. Bacteriol.*, **24**, 3 (1921).
8. FERRARO, F. M., AND APPLEMAN, M. D. Microbiology of frozen orange concentrate. IV. Further studies of enterococci in frozen orange concentrate. *Bacteriol. Proc., 56th Gen. Meeting*, p. 26 (1956).
9. FRANCE, K. L., AND FULLER, J. E. Coliform bacteria and streptococci in swimming pool water. *Am. J. Public Health*, **30**, 1059 (1940).
10. HAENEL, H., AND MUELLER-BUETHOW, W. Vergleichende quantitative Untersuchungen ueber Keimzahlen in den Faeces des Menschen und einigen Wirbeltiere. *Zentr. Bakteriol., I. Abt.*, **0.**, 167, 123 (1956).
11. HAJNA, A. A., AND PERRY, C. A. Comparative study of presumptive and confirmative media for bacteria of the coliform group and for fecal streptococci. *Am. J. Public Health*, **33**, 550 (1943).
12. HUCKER, G. J., BROOKS, R., AND EMERY, A. J. The source of bacteria in processing and their significance in frozen vegetables. *Food Technol.*, **6**, 147 (1952).
13. HUNTER, A. G. Uses and limitations of the coliform group in sanitary control of food production. *Food Research*, **4**, 531 (1939).
14. INGRAM, M., AND BARNES, E. M. Streptococci in pasteurized canned hams. *Ann., Inst. Pasteur de Lille*, **7**, 101 (1955).
15. KAPLAN, M. T., AND APPLEMAN, M. D. Microbiology of frozen orange concentrate. III. Studies of enterococci in frozen concentrated orange juice. *Food Technol.*, **6**, 167 (1952).
16. LAKE, D. E., DEIBEL, R. H., AND NIVEN, C. F., JR. The identity of *Streptococcus faecium*. *Bacteriol. Proc., 57th Gen. Meeting*, p. 13, (1957).
17. LARKIN, E. P., LITSKY, W., AND FULLER, J. E. Fecal streptococci in frozen foods. I. A bacteriological survey of some commercially frozen foods. *Appl. Microbiol.*, **3**, 98 (1955).
18. LEININGER, H. V., AND MCCLESKEY, C. S. Bacterial indicators of pollution in surface waters. *Appl. Microbiol.*, **1**, 119 (1953).

19. LITSKY, W., ROSENBAUM, M. J., AND FRANCE, R. L. A comparison of the most probable numbers of coliform bacteria and enterococci in raw sewage. *Appl. Microbiol.*, **1**, 247 (1953).
20. MALLMAN, W. L. A new yardstick for measuring pollution. *Sewage Works J.*, **12**, 875 (1940).
21. MALLMAN, W. L., AND LITSKY, W. Survival of selected enteric organisms in various types of soil. *Am. J. Public Health*, **41**, 38 (1951).
22. MALLMANN, W. L., AND SELIGMANN, E. B. A comparative study of media for the detection of streptococci in water and sewage. *Am. J. Public Health*, **40**, 286 (1950).
23. MEDREK, T. F., AND LITSKY, W. The comparative incidence of coliform bacteria and enterococci in virgin soil. *Bacteriol. Proc., 57th Gen. Meeting*, p. 141 (1957).
24. OSTROLENK, M., AND HUNTER, A. C. The distribution of enteric streptococci. *J. Bacteriol.*, **51**, 735 (1946).
25. RANDALL, J. S. The sanitary significance of coliform bacilli in soil. *J. Hyg. (Camb.)*, **54**, 365 (1956).
26. SHERMAN, J. M. The streptococci. *Bacteriol. Rev.*, **1**, 3 (1937).
27. SKADHAUGE, K. Studies on enterococci with special reference to the serological properties. Copenhagen: Einar Munksgaards. (1950). (Cited by Barnes, E. M.; *vide supra*).
28. VAUGHN, R. H., MURDOCK, D. I., AND BROKAW, C. H. Microorganisms of significance in frozen citrus products. *Food Technol.*, **11**, 92 (1957).
29. WINTER, C. E., AND SANDHOLZER, L. A. Isolation of enterococci from natural sources. *J. Bacteriol.*, **51**, 588 (1946).

RIBOFLAVIN, NIACIN AND THIAMINE IN APALACHICOLA BAY OYSTERS [a, b]

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The concentration of certain constituents of oysters has been known to be affected by season, salinity of the water, and environmental temperature. Chipman *(3)* has observed that the glycogen content of oysters is almost completely exhausted after spawning and is replenished during fall and winter months. Better growth and meat yields were observed by Butler *(2)* in oysters grown in the colder water of the Chesapeake Bay area as compared with those grown in the Gulf of Mexico. Medcof and Needler *(8)* found that oysters more nearly filled the volume of the shall cavity at temperatures between 15° and 20° C. Above this temperature, they may continue to gain in size, until spawning causes a loss of tissue.

The main purpose of the present investigation was to follow any seasonal variations which might occur in the amounts of riboflavin, niacin, and thiamine in Southern oysters. At the same time it seemed worthwhile to study the relation between amounts of certain vitamins of the B complex and other factors such as dry matter and protein.

MATERIALS AND METHODS

Oysters *(Crassostrea Virginica)* obtained from Cat Point Reef in Apalachicola Bay, Florida, were used for the assays. Samples were obtained at approximately monthly intervals from February, 1955, through August, 1956. Most of the samples were tonged, packed in ice, and immediately transferred to the laboratory. Additional lots were obtained from a commercial packing house in Apalachicola. In these cases the oysters had been tonged the previous day and were packed in ice in the shell or shucked and packed into metal friction top cans.

In the laboratory the oysters were shucked and washed by suspending them in a large container of tap water for approximately 30 seconds. After each lot of oysters had been well mixed, duplicate 200 g. aliquots were homogenized in a Waring blender for determinations of total solids, riboflavin, niacin, and thiamine. When it was impossible to complete all vitamin assays immediately after sampling, the oyster homogenates were placed in pint fruit jars, sealed with tape and stored at −15° C.

Total solids in the oyster homogenates were measured by using a modification of a method developed by Price and Traynor *(9)*. Quadruple samples of 3 to 5 g. each were dried for 6 to 8 hours in a mechanical convection oven at 100° C. Nitrogen was determined by the Kjeldahl method.

Niacin and riboflavin in oysters were determined microbiologically by using *Lactobacillus arabinosus*, strain 17-5, and *Lactobacillus casei*, respectively. The basal medium and procedure described in *Methods of Vitamin Assay (1)* were used in the niacin assays. Riboflavin was determined by the use of the Landy and Dicken *(6)* medium, modified by the addition of para amino benzoic acid. Jansen's thiochrome method as modified by Conner and Straub *(4)* was used for estimating thiamine.

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RESULTS AND DISCUSSION

Data on total solids, niacin, riboflavin, and thiamine in fresh, raw, washed oysters are given in Table 1. Each value in the table represents an average of duplicate samples from one lot of oysters, with the following exceptions. Three lots were sampled in February, 1955, and April, 1956. Oysters were sampled twice in April, 1955, January, February, March and July, 1956.

TABLE 1

Vitamin content, and per cent total solids in raw oysters

Month	Per cent solids	Niacin		Riboflavin		Thiamine	
		Wet	Dry	Wet	Dry	Wet	Dry
		Mg./100 g.	*Mg./100 g.*	*Mg./100 g.*	*Mg./100 g.*	*Mg./100 g.*	*Mg./100 g.*
1955							
February	17.5	1.59	9.4	0.20	1.12	.....	.....
April	18.7	2.05	10.8	0.20	1.09	.....	.....
May	15.7	1.68	10.6	0.09	0.57	.....	.....
June	11.5	1.51	13.3	0.08	0.73	.....	.....
July	16.2	2.52	15.6	0.12	0.74	.....	.....
August	13.4	1.99	14.9	0.12	0.89	.....	.....
September	15.4	2.45	15.9	0.06	0.41	.....	.....
October	21.8	3.34	15.4	0.27	1.22	0.08	0.36
November	13.2	1.41	10.6	0.24	1.83	0.08	0.61
December	13.9	1.54	11.1	0.26	1.87	0.12	0.85
1956							
January	15.1	1.47	9.7	0.12	0.75	.....	.....
February	11.7	1.41	11.9	0.10	0.83	0.10	0.82
March	16.0	2.07	13.1	0.21	1.28	0.16	0.94
April	16.0	2.14	13.6	0.27	1.64	0.18	1.00
May	15.1	2.12	14.1	0.23	1.51	0.12	0.81
June	14.1	1.95	13.9	0.23	1.66	0.12	0.86
July	15.9	2.13	13.3	0.28	1.77	0.13	0.79
August	13.3	2.05	14.7	0.20	1.49	0.10	0.72
Average	15.2	1.97	12.9	0.18	1.19	0.12	0.78

Variations in amounts of dry matter of oysters prepared in the manner described showed no seasonal trend. The lowest value, 11.5%, represented oysters assayed in June, 1955, whereas the highest value, 21.8%, was observed in October of the same year. Shucking and washing techniques may have been responsible for some variations in amount of water lost from the oysters in spite of all precautions. Since sampling was made on a weight basis, changes in size of individual oysters might affect the proportion of constituents, assuming that this change was related to glycogen storage rather than amount of active tissue.

Assays of oyster liquor showed that only traces of the 3 vitamins were present. This finding suggested that some of the variation in vitamin content in the fresh oysters might be a reflection of degree of hydration of the tissue. Calculation of the vitamin values on the dry basis did not remove the differences between the samples, although the highest amount of dry matter, 21.8%, corresponded with the highest niacin value, 3.34 mg., and the second highest riboflavin value, 0.27 mg. per 100 g.

Niacin concentrations in oysters showed no decline during the summer months. During the period of the investigation the amounts of niacin ranged from 1.41 to 3.34 mg. per 100 g. of fresh oysters. On the dry basis the range

was from 10.6 to 15.9 mg. per 100 g. dry material, with some of the highest values occurring during summer and early fall months.

Riboflavin values fluctuated with the various samples, with a range of 0.06 to 0.29 mg. per 100 g., fresh, or 0.57 to 1.87 mg. per 100 g. on the dry basis. Thiamine values were of the same order, 0.08 to 0.18 and 0.36 to 1.00 mg. per 100 g. on the fresh and dry materials, respectively.

At the beginning of the survey, samples of oyster homogenates were frozen for later analysis with the assumption that thiamine is stable under freezing conditions. Upon analysis of these samples it was found that the thiamine content was so low that it was difficult to obtain valid readings on the photofluorometer. Since these findings were not in line with the assays of fresh oysters, thiamine values for only the fresh tissues are included in Table I.

The possibility of enzymatic destruction of thiamine was investigated in several experiments on oysters before and after frozen storage. As is shown in Table 2, oyster homogenates lost from 63 to 67% of the original content of

TABLE 2

Thiamine losses during frozen storage

Treatment of oysters	Storage at −15° C. in weeks	Thiamine mg./100 g. fresh basis	Per cent loss
Homogenate	0	.08	
Homogenate	3	.03	63
Homogenate + .04 mg. thiamine/100 g	3	.04	67
Homogenate	15	.03	63
Whole	15	.06	24

this vitamin during frozen storage of only three weeks. When the oysters were left whole, there was only a 24% loss of thiamine after 15 weeks of frozen storage. A sample of oyster homogenate which had been cooked contained .14 mg. thiamine per 100 g. before freezing and .11 mg. per 100 g. three weeks later. The decrease of only 20% in this case may be the result of inactivation of an enzyme by heating.

Various reports of assays on thiaminase in fish and shellfish have shown that oysters contain very small amounts of the enzyme. Neilands *(7)* found only a slight loss of added thiamine using *Ostrea Edulis*. Fujita *(5)* stated that thiaminase was scarcely detected in Japanese oysters. The fact that the losses of thiamine observed in this study occurred only in the homogenized sample suggests that the active material is bound in the intact oyster. Since the maceration of tissue is not a common practice in handling oysters, the possibility of a thiamine loss from this source is minimized.

Nitrogen determinations were made on some of the samples in an attempt to learn whether or not fluctuations in vitamin content were caused by dilution of the tissues with varying amounts of water and glycogen. As is shown in Table 3 there was no obvious relationship between amounts of these vitamins and amounts of nitrogen. The oysters ranged from 42 to 57% protein, on the dry basis, with as many high values among the summer samples as those taken when oysters were considered to be of higher quality.

SUMMARY

Oysters from Apalachicola Bay were assayed for niacin, riboflavin, and total solids at monthly intervals from February, 1955, through August, 1956. Thiamine determinations were also made beginning with October, 1955. A

TABLE 3

Protein content and vitamins per g. nitrogen in oysters

Date	Per cent protein, N x 6.25		Niacin mg./g. nitrogen	Riboflavin mg./g. nitrogen	Thiamine mg./g. nitrogen
	Fresh	Dry			
1955					
February	7.2	42	1.54	0.18	.....
April	8.6	53	1.29	0.09	.....
May	8.1	51	1.30	0.07	.....
June	5.8	51	1.64	0.09	.....
July	9.0	53	1.87	0.09	.....
August	7.8	56	1.67	0.10	.....
September	8.9	57	1.74	0.05	.....
October	8.0	56	1.72	0.14	0.04
November	5.8	50	1.32	0.23	0.08
December	7.3	52	1.33	0.23	0.10
1956					
January	7.7	47	1.25	0.08	0.08
February	5.1	42	1.84	0.13	0.13
March	7.8	48	1.30	0.10	.....
April	9.8	48	1.83	0.22	0.13
May	7.4	47	1.88	0.20	0.11
Average	7.6	50	1.49	0.13	0.10

portion of the samples were analyzed for nitrogen and per cent protein was calculated using the factor 6.25.

During the summer months, when oysters are considered to be of poor quality, there was no obvious change in the levels of niacin, riboflavin, or thiamine. In fact, niacin values appeared to be slightly higher during this season. Averages for the entire period in mg. per 100 g. fresh oysters were: niacin, 1.97; riboflavin, 0.18; thiamine, 0.12. Although there were large differences in amounts of total solids and protein, these constituents showed little relation to the concentrations of the three vitamins studied.

Thiamine values of oyster homogenates decreased rapidly during frozen storage. Much smaller losses occurred when the oysters were whole or had been heated prior to freezing.

LITERATURE CITED

1. Association of Vitamin Chemists. *Methods of Vitamin Assay*, 2nd ed., 1951. Interscience Publishers, Inc., New York, p. 180.
2. Butler, P. A. Oyster growth as affected by latitudinal temperature gradients. *Com. Fish. Rev.*, **15**, No. 6, 7 (1953).
3. Chipman, W. A. Seasonal variations in oyster fattening. *Atl. Fisherman*, **29**, 16, 40 (1948).
4. Conner, R. T., and Straub, G. T. Determination of thiamine by the thiochrome reaction. *Ind. Eng. Chem. Anal. Ed.*, **13**, 38 (1941).
5. Fujita, A. Thiaminase. *Advances in Enzymology*, **15**, 389 (1954).
6. Landy, M., and Dicken, D. M. A microbiological assay method for six B vitamins using *Lactobacillus casei* and a medium of essentially known composition. *J. Lab. and Clin. Med.*, **27**, 1086 (1942).
7. Neilands, J. B. Thiaminase in aquatic animals of Nova Scotia. *J. Fish. Research Bd. of Canada*, **7**, 94 (1947).
8. Medcof, J. C., and Needler, A. W. H. The influence of temperature and salinity on the condition of oysters *(Ostrea virginica)*. *J. Fish. Research Bd. of Canada*, **5**, 253 (1941).
9. Price, D. C., and Traynor, J. P. Report of solids in raw oysters. *J. Assoc. Off. Agr. Chem.*, **36**, 608 (1953).

SOFTENING ENZYME ACTIVITY OF CUCUMBER FLOWERS FROM NORTHERN PRODUCTION AREAS [a]

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When pectic substances in the middle lamella of pickling varieties of cucumbers are hydrolyzed by pectic enzymes, softening of the tissue results. Previous studies *(3, 5)* have demonstrated that a pectin-splitting enzyme, similar in behavior to polygalacturonase, is chiefly responsible for the softening of salt-stock cucumbers cured under commercial conditions. The enzyme was found to be active in brines from numerous commercial brining stations and its activity correlated with the softening of the salt-stock. It was further shown that a purified form of the enzyme could readily deteriorate the firmness of sound salt-stock cucumbers. Additional studies *(4, 7, 9)* have since demonstrated that cellulolytic enzyme systems are also present in curing brines and may contribute in part to the total softening action of brined material.

It has been established *(7, 8)* that the pectinolytic and cellulolytic enzyme systems are introduced into the curing vats by way of the partially dried cucumber flowers that remain attached to the cucumbers, particularly the small-sized fruit (see Figure 1, right). In addition, microbiological studies strongly point to mold growth in the cucumber flowers as the actual causative agent responsible for the softening enzyme activity.

Numerous brining experiments covering several seasons in two important production areas of the South *(7)* appear to substantiate the basic findings on the origin of softening enzymes in cucumber brines. For example, brine samples from vats filled with small-sized cucumbers (1 to 1⅛ in. dia.) that had a high percentage of flowers, either retained by the cucumbers or added experimentally, were shown to have high enzyme activity and usually the cured salt-stock was either soft or definitely inferior in firmness. However, when the cucumbers were deflowered by hand, the brine samples were low in enzyme activity and the cured stock was exceptionally firm.

More recently, Bell and Etchells *(1)* have found that pectinolytic and cellulolytic enzymes are inhibited by a water soluble fraction of Scuppernong grape leaves *(Vitus rotundifolia)*. Furthermore, cucumber brining tests have demonstrated that the pectinolytic and cellulolytic enzyme systems of cucumber flowers were effectively reduced in activity by use of a crude extract of Scup-

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[a] This study was carried out under a cooperative project with the Department of Horticulture of the North Carolina Agricultural Experiment Station.

[b] One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

[c] Journal article No. 2102, Michigan Agricultural Experiment Station.

pernong leaves *(2)*. The inhibition of the softening enzymes resulted in an increased firmness of the fermented, brine-cured cucumbers.

Softening can, at times, be a serious problem in northern brining areas as well as in other sections of the country. Etchells *(6)*, during the Fall of 1955, tested the firmness of 89 vats of commercially brined salt-stock from 4 brining stations located in Michigan and Indiana. Examination of the stock for firmness demonstrated that approximately 25% of the material as of October 17, 1955 was less than firm . . . also, of the 89 vats examined, stock from 7 had lost more than 50% in firmness and were definitely soft. Although this was the only instance of commercial softening observed first-hand during the 1955 season in northern brining areas, reports from packers have since indicated that loss of firmness of salt-stock, to a varied degree, may have been widespread in Indiana, Michigan, and Wisconsin that year.

The present investigation was undertaken chiefly as an exploratory survey to determine the nature and extent of softening enzyme activity of cucumber flowers collected in northern production areas; the study also provided the opportunity to compare the values obtained for pectinolytic and cellulolytic activity with those determined earlier for flower samples collected from southern areas (i.e., brining stations located in North Carolina and Texas). It was an interesting coincidence that the study on the softening enzyme activity of cucumber flowers from the northern production area was made during a season (1955) which provided direct evidence of enzymatic softening of commercially brined cucumbers in that area.

MATERIALS AND METHODS

Collecting flower samples and flower retention data. During August 16–25, 19 samples of retained cucumber flowers (Figure 1, Right) obtained from No. 1 size (1 to 1 1/16 in. dia.) stock were collected at 17 brining stations and 1 receiving station located in Michigan, Wisconsin, Indiana, and Ontario (Figure 2). Eight additional samples, consisting of freshly opened flowers (Figure 1, Left) were removed from cucumber ovaries in 8 commercial fields located in Michigan and Wisconsin. The brining stations and fields visited represented operations by 12 pickle companies and were selected over a wide area to give geographical coverage. Each sample was usually composed of 100 individual flowers; these were removed from the cucumbers or ovaries with forceps and put in polyethylene freezer-bags. Each sample was labelled and immediately frozen with dry ice and stored at approximately −10° C. until analyzed for enzyme activity. Additional information was gathered at each brining station as to the varieties planted and, the estimated flower retention on No. 1 size cucumbers. The latter was obtained by counting the number of retained flowers per 100 cucumbers; the final estimate was based on samples from 4 to 6 bushels of stock that had been machine graded.

Softening enzyme extraction and analysis. Each flower sample was weighed, counted, and placed in a Waring blender[4] with 100 ml. of 2% NaCl, blended for 1 minute, then filtered and the clear flower extract dialyzed against water for 4 hours using 0.75 in. dia. cellophane tubing. Viscometric methods were used to determine the pectinolytic and cellulolytic (Cx) enzyme activities; these have been given in detail by Bell *et al. (4)* and 100 units of pectinolytic activity equals 50% loss in viscosity in 20 hours of a 1.0% sodium polypectate solution, buffered with citrate at pH 5.0, at 30° C. Cellulolytic activity units are equal to the same conditions as above except 1.0% sodium carboxymethylcellulose is used as the substrate.

[4] It is not the policy of the Department of Agriculture to recommend the products of one company over similar products of any other company. The name supplied herewith is furnished for your convenience and information.

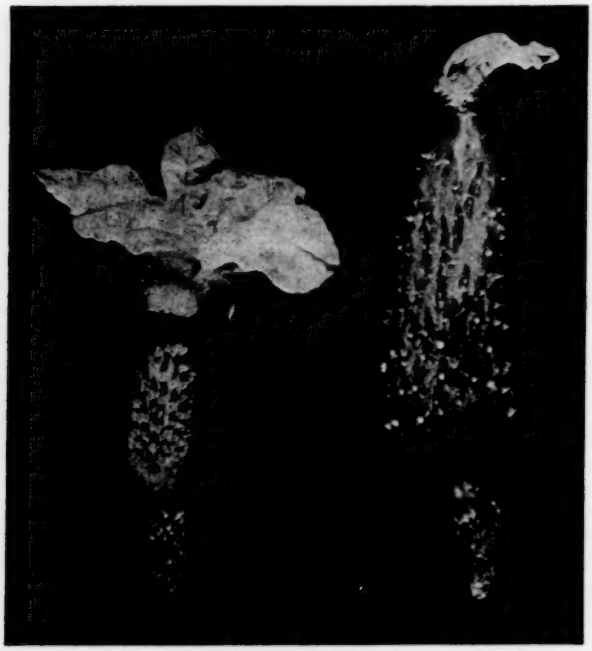

Figure 1. Left: Opened pistillate flower typical of samples collected from the growing cucumber plant in commercial fields.
Right: Partially dried flower on a small sized (No. 1) cucumber; this retained flower is typical of samples removed from 1 to 1 1/16" diameter cucumbers at brining stations. About actual size.

RESULTS AND DISCUSSION

Flower retention on cucumbers. It has been observed *(8)* that flower retention of commercially harvested, machine graded No. 1 size cucumbers

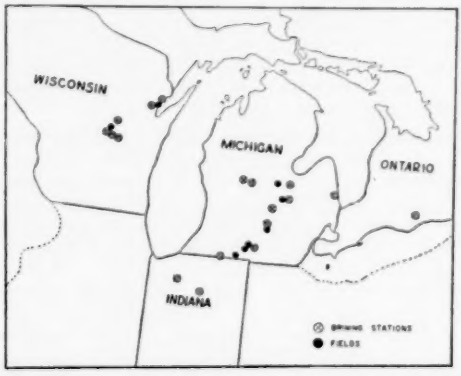

Figure 2. Geographical location of brining stations and commercial fields where cucumber flower samples were collected during 1955 harvest.

grown in two important production areas in the South can vary extensively during the intake season. Retention was usually found to be highest (70 to 80%) during the first part of the harvest, whereas during the latter part it was negligible (5 to 10%). Furthermore, flower retention was greater on smaller fruit than on the larger.

The cucumber fields visited during the northern survey were in their third to fourth week of production and the crop conditions varied greatly; ranging from lush, vigorous vines with heavy production in certain parts of Michigan to less vigorous vines suffering from high temperatures and lack of rain in the principal growing sections of Wisconsin. Information on the percentage of flowers retained by the different cucumber varieties examined at northern brining stations is given in Table 1; also included are flower weights. The

TABLE 1

Estimated flower retention, weight, and enzyme activity of cucumber flowers from different varieties sampled at northern brining stations during Aug. 16-25, 1955

Brining station	Variety[4]	Estimated retention of flowers	Average flower weight	Pectinolytic activity (per flower)	Cellulolytic activity (per flower)
code		%	*grams*	*units*	*units*
Michigan					
WP	SMR-12-12	26	0.021	97	547
LT	MR-25	10	0.024	98	707
MA	SR-6	8	0.022	122	654
LA[1]	SR-6	4	0.017	33	677
NL	SR-6	5	0.015	59	554
SG	SR-6	11	0.019	55	734
ED	SR-6	8	0.022	33	780
LV	SR-6	7	0.016	165	488
CR	SR-6	3	0.031	37	340
Wisconsin					
AB[1]	SR-6	14	0.031	80	246
OC	SR-6	6	0.025	193	654
WA	SMR-12-12	47	0.027	240	487
SL-1	SMR-12-12	29	0.028	233	407
SL-2[2]	SMR-12-12	25	0.031	31	213
WU	SMR-12-12	40	0.027	78	507
BR	SR-6 and SMR-12-12	12	0.025	147	547
Indiana					
NJ[3]	MR-25	12	0.029	308	2,199
MC[3]	MR-17	2	0.040	201	1,421
Ontario					
WF	MR-17 and Chicago pickling	3	0.021	60	760
Average			0.0238	120	680

[1] Receiving and grading station only.
[2] Represents a second sample from Station SL collected September 4.
[3] Material from these 2 stations was shipped to Chicago by truck on August 20 and sampled August 22.
[4] SR (= scab resistant) and SMR (= scab and mosaic resistant) represent University of Wisconsin introductions; MR (= mosaic resistant) represents Ohio State University introductions.

latter show that the average station flower weighed 0.0238 g. with a range from 0.015 to 0.040 g.

The most widely grown cucumber varieties in the areas visited in Michigan and Wisconsin were SR-6 and SMR-12-12 respectively. Flower retention values for varieties at all stations ranged from 2 to 47% with 8 of the stations above the 10% level; also, 4 of these were above 25%. Retention estimates from the 5 stations receiving and grading variety SMR-12-12 ranged from 25 to 47% (av. 33%). In contrast, only 2 of the 9 stations with variety SR-6 gave retention values above 10% and the range for all lots of this variety examined was 3 to 14% (av. 7%). Based on this information, it appears likely that flower retention estimates for the recently introduced variety SMR-12-12 were generally much higher than those for SR-6. Four of the five stations where SMR-12-12 was observed for flower retention were in Wisconsin where hot, dry weather was prevailing. Such unfavorable growing conditions may have contributed to flower retention of this variety. However, subsequent observations by northern packers during the 1956 season on these two varieties tend to substantiate the varietal difference indicated above.*

Enzyme activity of flowers from stations. The pectinolytic and cellulolytic activities of retained cucumber flowers collected at southern brining stations in 1953 have previously been presented *(7)*. However, summarized results based on more recent southern studies—1954 and 1955—are reported herein for comparison with the 1955 northern observations because improved technics *(4)* for measuring softening enzyme activity were in use for material collected in the two areas during the particular years mentioned.

The pectinolytic activity of 16 flower samples obtained from southern areas ranged from 34 to 185 units (av. 88) calculated on a per flower basis. These flowers, when added to experimental vats of No. 1 size cucumbers in quantities equal to 100% retention, reduced the firmness of the cured as much as 50 to 75% over that of deflowered controls. The pectinolytic activity values of the 19 flower samples collected from northern brining stations (Table 1) show a range from 31 to 308 units (av. 120).

The results for cellulolytic activity of flowers from southern and northern areas parallel those for pectinolytic activity. The cellulolytic activity of the 16 flower samples from southern areas ranged from 477 to 1,191 units (av. 782); the values for northern area flowers (Table 1) ranged from 213 to 2,199 units (av. 680). A rather wide variation of enzyme activity of flower samples was observed in both northern and southern production areas. It is important to mention that, based on previous experience, such variation in enzyme activity of southern flower samples from the same stations, may occur not only between seasons, but also for given periods within the same season.

These results indicate that retained cucumber flowers from northern production areas possess a potential softening enzyme activity equal to that of flowers from southern areas and which, if introduced into curing vats in sufficient amounts under suitable conditions, could reduce the firmness of brined material in a manner previously demonstrated for cucumbers brined

* Plant breeders at several State Agricultural Experiment Stations (North Carolina, Arkansas, Michigan, and Wisconsin) have expressed considerable interest in studying the factors responsible for flower retention in pickling cucumbers and are evaluating their new lines for this characteristic.

under southern conditions. In this connection, it will be recalled that enzymatic softening of commercially brined cucumbers was observed first-hand in October 1955, at northern brining stations in the same general area visited about 2 months earlier (August) during the survey.

Enzyme activity of field flowers. The enzyme activity of 8 samples of flowers removed from cucumber ovaries in fields located in Michigan and Wisconsin are presented in Table 2 together with the flower weights. The average weight of a freshly opened flower was found to be 0.281 g.; this was about 12 times the weight of a individual, partially dried flower removed from No. 1 size stock at the brining stations. The pectinolytic activity of flowers from fields ranged from 350 to 1,737 units (av. 875) calculated on a per flower basis; the cellulolytic activity ranged from 103 to 221 units (av. 151).

TABLE 2

Weight and enzyme activity of cucumber flowers from different varieties sampled from ovaries in 8 commercial fields located in Michigan and Wisconsin

Location of field	Variety	Average flower weight	Pectinolytic activity (per flower)	Cellulolytic activity (per flower)
code		*grams*	*units*	*units*
Michigan				
BN	MR-25	0.303	1737	140
LT	SMR-12-12	0.200	980	124
LT	MR-25	0.305	956	103
MA	SR-6	0.294	1012	132
NL	SR-6	0.366	1023	221
SG	SR-6	0.395	350	197
Wisconsin				
OC	Maine No. 2 and SR-6	0.219	511	160
WA	SMR-12-12	0.168	429	129
Average		0.281	875	151

Considering all samples collected from each source (brining stations and fields) the flowers removed from the ovaries in the fields had about 7 times the pectinolytic activity as compared to those removed from the No. 1 size fruit being delivered to the brining stations. In the case of cellulolytic activity, the situation was somewhat the reverse; here, the station flowers had about 4.5 times more actitvity than field flowers.

SUMMARY

In general, the exploratory findings reported herein indicate that retained cucumber flowers from northern production areas—such as Michigan, Wisconsin and Indiana—possess a potential softening enzyme activity (pectinolytic and cellulolytic) equal to that of flowers from southern areas. Furthermore, it seems most likely that the softening enzyme concentration of northern flowers was such that, if sufficient amounts were introduced into curing vats under suitable conditions, they could reduce the firmness of brined material in a manner previously demonstrated for cucumbers brined under southern conditions.

Acknowledgments

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LITERATURE CITED

1. Bell, T. A., and Etchells, J. L. Pectinase inhibitor in grape leaves. *Bot. Gaz.* (In Press, 1958).
2. Bell, T. A., and Etchells, J. L. Inhibition of pectinolytic and cellulolytic enzymes in cucumber fermentations by Scuppernong grape leaves. *Food Technol.* (In Press, 1958).
3. Bell, T. A., Etchells, J. L., and Jones, I. D. Softening of commercial cucumber salt-stock in relation to polygalacturonase activity. *Food Technol.*, **4**, 157 (1950).
4. Bell, T. A., Etchells, J. L., and Jones, I. D. A method for testing cucumber salt-stock brine for softening activity. U. S. Dept. Agr., *ARS-72-5*, 15 pp. (1955).
5. Demain, A. L., and Phaff, H. J. Cucumber curing: softening of cucumbers during curing. *J. Agr. Food Chem.*, **5**, 60 (1957).
6. Etchells, J. L. (Unpublished data, 1955.)
7. Etchells, J. L., Bell, T. A., and Jones, I. D. Studies on the origin of pectinolytic and cellulolytic enzymes in commercial cucumber fermentations. Presented at the 14th Annual Meeting, Institute Food Technologists, Los Angeles, Calif. (1954). *Food Technol.*, **9**, No. 3, 14 (1955). (Abst.)
8. Etchells, J. L., Bell, T. A., and Jones, I. D. Cucumber blossoms in salt-stock mean soft pickles. *Research and Farming.* (N. C. Agr. Expt. Sta.) **13**, (1–4), 14 (1955).
9. Jansen, E. F., and Seegmiller, C. G. (Personal communication, 1954).

EFFECTS OF RADIATION ON HEMATIN COMPOUNDS*

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Knowledge of the radiation chemistry of hematin compounds is important both for an understanding of the changes induced during radiation preservation of meats and for a better understanding of the mechanism of radiation damage to animals, plants and bacteria. The hematin pigments which give meat its characteristic color are variously affected during irradiation *(5, 6, 7, 9)*. Fresh meats irradiated at low doses in an oxygen atmosphere develop brownish discoloration due to the formation of metmyoglobin. Fresh meats irradiated in a nitrogen atmosphere develop a brighter coloration caused by the regeneration of oxymyoglobin in the radiation dose range of 0.1×10^6 to 4×10^6 rep *(16)*. In cooked meats the normal brown colored denatured globin hemichrome is converted to the red colored denatured globin hemochrome by irradiation at 2×10^6 rep *(15)*. Although not a practical problem it is interesting that the irradiation of meat extracts produces "green" compounds indicating oxidation of the porphyrin ring *(5)*.

The irradiation of hematin compounds is known to cause the following important changes. Spectral absorption studies showed that X-irradiation of oxyhemoglobin and oxymyoglobin solutions altered simultaneously the protein, the valence state of iron and the porphyrin ring *(1, 2)*. The oxidation of the iron and the destruction of porphyrin were proportional to dose. A number of investigators *(2, 10, 14)* have studied the radiation induced reduction of ferricytochrome c to ferrocytochrome c. The appearance of a chromophore at 610 mμ in irradiated cytochrome c has been interpreted by some *(10)* to indicate the formation of porphyrin c and by others *(2)* to indicate the opening of a methylene bridge in the porphyrin and the formation of an oxyporphyrin compound. Irradiation of methemoglobin, hemin and cyanide hemichrome also gave reduction to the corresponding hemoglobin, heme and cyanide hemochrome *(2)*.

In spite of considerable scientific activity in the radiation chemistry of hematin compounds, our knowledge remains relatively meager. Many of the important hematin compounds have not been radiated and in this study the effect of relatively large doses of gamma radiation on a variety of hematin compounds will be surveyed. More conclusive evidence is needed for the formation of oxyhemoglobin by irradiation of methemoglobin. In this study conditions were sought for optimum conversion of methemoglobin to oxyhemoglobin. The formation of the chromophore absorbing at 610 mμ in the irradiated cytochrome c is interesting in itself and further study of this should increase our understanding of similar "green" compounds formed by the oxidation of the porphyrin of the hemochrome pigments of cooked cured meats.

* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

In this study optimum conditions for the production of the chromophore absorbing at 610 mμ was sought and the reaction resulting in this absorbance is explained.

EXPERIMENTAL

The hematin compounds irradiated for these studies are listed in Table 1 with their source of method of preparation, their concentrations, the buffer and pH of the solution, the atmosphere, and the radiation dose. All of the hematin compounds were sealed in 100 ml. polyethylene bottles after degassing and gassing three times with nitrogen or oxygen. Polyethylene bottles offered the advantages of being relatively inert, nonbreakable, and easily punctured by hypodermic needles to anaerobically remove samples. The polyethylene bottles were sealed in no. 2 cans. The samples were kept in the frozen state during transportation to and from the Materials Testing Reactor and storage prior to analysis. For irradiation the samples were adjusted to 0° C. and placed in an insulated chamber. The samples were irradiated at the Materials Testing Reactor, Arco, Idaho to the doses shown in Table 1.

The complete visible spectra of all the irradiated hematin compounds were examined, and when interesting changes were found, other studies were made. Destruction of the hematin nucleus of the irradiated hemoproteins was measured by alkaline pyridine hemochrome method. Sulfhydryl groups were measured by amperometric titration *(3)*. Autoxidizable cytochrome c was determined by the method of Tsou *(18)*. Catalase activity was determined manometrically.

RESULTS AND DISCUSSION

Methemoglobin. Table 2 and Figure 1 give results of two experiments directed toward obtaining maximum conversion of methemoglobin to oxyhemoglobin with minimum destruction of the hemoproteins. At 6 x 10^{-4}M methemoglobin, good conversion to 20% oxyhemoglobin was obtained as seen in Figure 1-1. Spectral absorbance maxima in the regions of 535 mμ and

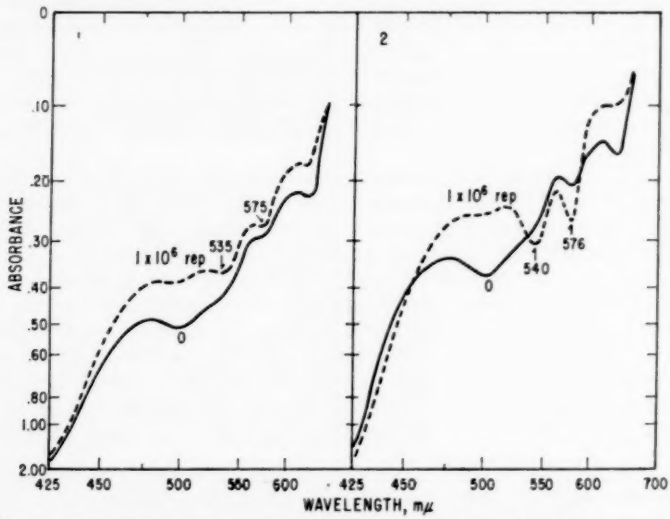

Figure 1. The absorption spectra of irradiated methemoglobin and the controls.
1. 6 x 10^{-4}M methemoglobin
2. 2 x 10^{-4}M methemoglobin

TABLE 1

Preparation and irradiation of hematin compounds

Hematin compound	Source	Concentration	Buffer	pH	Atmosphere	Irradiation doses
		M x 10⁴				*r.e.p. x 10⁻⁶*
Methemoglobin	Oxidation of oxyhemoglobin with ferricyanide followed by dialysis	6	0.1M phosphate	7.0	N_2	0.1, 0.25, 0.5, 1
		2	0.01M phosphate	7.0	N_2	0.1, 1
Oxyhemoglobin	Purified from fresh cattle blood	2	0.01M phosphate	7.0	O_2	0.2, 2, 20
Hemoglobin	Reduction of methemoglobin with hydrosulfite	2	0.01M phosphate	7.0	N_2	0.2, 2, 10
Carboxyhemoglobin	Hemoglobin gassed with carbon monoxide	2	0.01M phosphate	7.0	N_2	0.2, 2, 10
Nitric oxide hemoglobin	1 mole hemoglobin plus 10 moles nitrite	2	0.01M phosphate	7.0	N_2	0.2, 2, 10
Hemiglobin cyanide	1 mole methemoglobin plus 2 moles cyanide	2	0.01M phosphate	7.0	N_2	0.2, 2, 10
Hemiglobin azide	1 mole methemoglobin plus 2 moles azide	2	0.01M phosphate	7.0	N_2	0.2, 2, 10
Denatured globin hemichrome	Alkali denatured oxyhemoglobin	2	0.1M hydroxide		N_2	0.2, 2, 10
Pyridine-denatured globin hemichrome	Denatured globin hemichrome plus pyridine	2	0.05M hydroxide		N_2	0.2, 2, 10
Cytochrome c	From Sigma Chemical Co.	2	0.01M phosphate	7.0	O_2	0.2, 2, 20
Cytochrome c	From Sigma Chemical Co.	2	0.01M phosphate	7.0	N_2	0.2, 2, 20
Cytochrome c	From Sigma Chemical Co.	0.2	0.01M phosphate	7.0	N_2	2
Cytochrome c	From Sigma Chemical Co.	2	0.01M phosphate	7.0	N_2	1, 2, 3, 5
Cytochrome c	From Sigma Chemical Co.	20	0.01M phosphate	7.0	N_2	2
Catalase	From Nutritional Biochemicals Co.	2	0.01M phosphate	7.0	O_2	2, 5, 20, 50
Catalase	From Nutritional Biochemicals Co.	2	0.01M phosphate		N_2	0.1, 0.2, 0.3, 0.5, 1, 2, 3, 20, 50
Hematin	From Armour & Co.	2	0.01M ammonium	9.0	O_2	0.2, 2, 20
Hematin	From Armour & Co.	2	0.01M ammonium	9.0	N_2	0.2, 2, 20

TABLE 2

Formation of oxyhemoglobin by irradiation of methemoglobin

Concentration of methemoglobin	Dose	Insoluble	Destruction of hematin nucleus	Sulfhydryl groups	Converted to oxyhemoglobin
				mole hemoglobin	
M x 10⁴	*r.e.p. x 10⁻⁴*	%	%		%
6	0	0	0	1.90	0
	0.5	0	0	.....	3
	1.0	16	5–10	1.19	20
2	0	0	0	2.88	0
	0.1	0	4	.....	0
	1.0	19	18	1.46	52

575 mμ are characteristic of oxyhemoglobin. At 2 x 10^{-4}M, what appears to be a maximum obtainable conversion of 50% was obtained as seen in Figure 1–2. Absorbance maxima at 540 mμ and 576 mμ are characteristic of oxyhemoglobin which has beta and alpha peaks at 540–542 mμ and 576–578 mμ, respectively. In these two experiments the measurements of solubility and the hematin nucleus show that these hemoproteins were not changed drastically in comparison to the amount of conversion to oxyhemoglobin. The sulfhydryl groups of hemoglobin are very labile to oxidation and during separation and oxidation to methemoglobin there was a decrease from four sulfhydryl groups to the initial value given in Table 2. Further decrease in sulfhydryl content was found upon irradiation and this probably was caused by the oxidizing effect of hydroxyl radicals. Both of these irradiated methemoglobin samples were subject to other reactions characteristic of authentic oxymyoglobin. They formed hemoglobin when reduced with sodium hydrosulfite. They oxidized readily with potassium ferricyanide and were completely converted back to methemoglobin. The oxyhemoglobin present exchanged with carbon monoxide to form carboxy hemoglobin. This exchange reaction with carbon monoxide is a good test for the identity of oxyhemoglobin. This conversion of methemoglobin to oxyhemoglobin may take place by the mechanism:

$$\text{methemoglobin} \xrightarrow{+\cdot OH} \text{ferrylhemoglobin} \xrightarrow{+\cdot OH} \text{oxyhemoglobin}$$

which has been proposed *(16)*.

Oxyhemoglobin. Study of the complete spectra of irradiated oxyhemoglobin and other chemical studies showed no unusual changes besides the destruction of this hemoprotein, the protein characteristic absorbance, the sulf-

TABLE 3

Irradiation destruction of oxyhemoglobin

Dose	Loss of oxyhemoglobin measured at		Denaturation of protein measured at 275 mμ	Destruction of hematin nucleus	Sulfhydryl groups
	577 mμ	412 mμ			mole hemoglobin
r.e.p. x 10⁻⁴	%	%	%	%	
0	0	0	0	0	2.98
0.2	15	....	2	3	2.51
2	95	96	89	66	
20	100	100	94	100	

hydryl groups and the hematin nucleus. Most significant was the fact that there was no production of methemoglobin; where as methemoglobin is noted when meat is irradiated in oxygen atmospheres. Although large destruction was found at 2×10^6 rep the remaining oxyhemoglobin retained some of its fundamental properties. The oxyhemoglobin irradiated 2×10^6 rep readily exchanged with carbon monoxide and was oxidized to methemoglobin by treatment with ferricyanide.

Hemoglobin. Hemoglobin is very labile to oxidation; these samples underwent some oxidation to methemoglobin during transport to and from the reactor and storage prior to analysis. The control sample contained some methemoglobin. The sample irradiated at 0.2×10^6 rep contained larger quantities of methemoglobin. The sample irradiated at 2×10^6 rep contained oxyhemoglobin. At 10×10^6 rep there was a complete loss of spectral characteristics. Apparently under these packaging conditions some of the hemoglobin was oxidized to methemoglobin and the irradiation at 2×10^6 rep converted the methemoglobin to oxyhemoglobin.

Carboxyhemoglobin. There were no spectral changes in carboxyhemoglobin when radiated at 0.2×10^6 and 2×10^6 rep. At 10×10^6 rep carboxyhemoglobin was precipitated.

Nitric oxide hemoglobin. The control sample of nitric oxide hemoglobin contained appreciable quantities of methemoglobin showing that it was oxidized during shipment to and from the reactor and subsequent storage. The samples irradiated at 0.2×10^6 rep and 2×10^6 rep showed complete reduction to nitric oxide hemoglobin and no radiation destruction. Irradiation at 10×10^6 rep caused loss of spectral characteristics and a total hematin pigment destruction of 56%. Excess nitrite is known to protect the cured meat pigment *(4)* and probably was a protector here.

Hemiglobin cyanide and Hemiglobin azide. There were no significant changes in hemiglobin cyanide and hemiglobin azide irradiated at 0.2×10^6 rep and 2×10^6 rep. At 10×10^6 rep both of these hemoproteins were precipitated.

Denatured globin hemichrome. Irradiation of denatured globin hemichrome at 2×10^6 rep gave a color change from tan to red indicating reduction to the hemochrome. Because of its oxygen lability no spectra were recorded. At 10×10^6 rep there was a loss of spectral characteristics and much of the color with a destruction of 62% of the denatured globin hemichrome.

Pyridine denatured globin hemichrome. There was no change in spectral absorption of pyridine denatured globin hemichrome when irradiated at 0.2×10^6 rep. At 2×10^6 rep there was essentially complete reduction to the corresponding hemochrome. This hemochrome readily autoxidized. At 10×10^6 rep all spectral characteristics of pyridine denatured globin hemichrome were lost.

Cytochrome c. The first experiment was a radiation of cytochrome c at 0.2×10^6, 2×10^6 and 20×10^6 rep in oxygen and nitrogen atmospheres. There was good reduction of the ferricytochrome c to ferrocytochrome c and the formation of a 608 mμ chromophore at 0.2×10^6 and 2×10^6 rep. In contrast to the orange color of ferricytochrome c and the pink color of ferrocytochrome c irradiation cytochrome c containing the 608 mμ chromophore was purple in color. Subsequent studies of cytochrome c irradiation were designed to learn conditions for maximum production of the 608 mμ chromophore and to establish its identity.

The results of this study are summarized in Table 4. At 2 x 10^{-4} M cytochrome c was destroyed as a function of the radiation dose. At constant radiation dose of 2 x 10^6 rep the amount of destruction of cytochrome c was a function of concentration. Maximum reduction of ferricytochrome c to ferrocytochrome c and maximum production of the 608 mμ chromophore were obtained when 2 x 10^{-4} M cytochrome c was irradiated at 2 x 10^6 rep. There was little change in autoxidizable cytochrome c as a function of radiation.

TABLE 4

The effects of irradiation of cytochrome c

Concentration of cytochrome c	Dose	Cytochrome c remaining	Reduction	Autoxidizable cytochrome c	Relative formation of the 608 mμ chromophore
M x 10⁴	*r.e.p. x 10⁻⁶*	%		%	
2	0	100	+	22	0
2	1	57	++	29	0.75
2	2	46	+++	20	1.00
2	3	21	++		0.72
2	5	1	+		0
20	2	62	+++		0.79
0.2	2	30	−		0

The formation of the 608 mμ chromophore was the most interesting result and was studied in further detail. The spectral absorbance of irradiated cytochrome c compared to the control sample is shown in Figure 2. Reduction or oxidation of radiated cytochrome c changed the absorption in the 608 mμ region only slightly. Assuming that the loss of cytochrome c gave rise to the 608 mμ chromophore a molecular extinction coefficient of 18,000 was calcu-

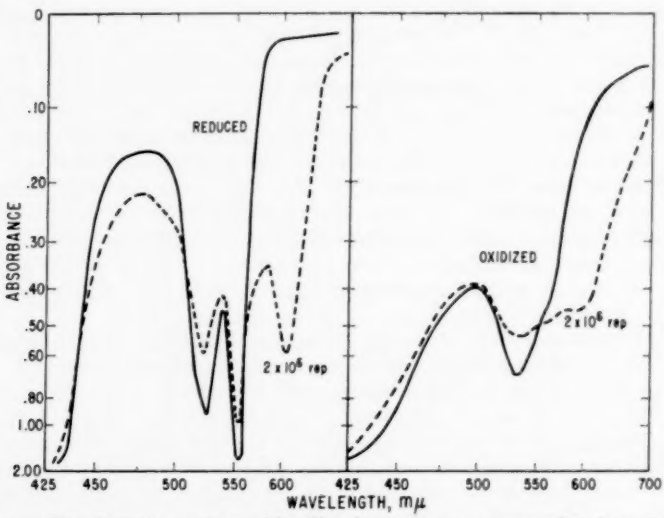

Figure 2. The absorption spectra of irradiated cytochrome c compared to its control.

lated for the 608 mμ chromophore. Mee and Stein *(10)* in a study of irradiation of cytochrome c suggested that this chromophore may be caused by the production of porphyrin c. Study of the solubility properties of the irradiated cytochrome c showed that the 608 mμ chromophore did not possess the solubility properties of porphyrin c *(8, 17)* and therefore this possibility was eliminated. Precipitation of irradiated cytochrome c and pure cytochrome c with trichloroacetic acid or acetone and resolution of the precipitated protein showed that the radiated cytochrome c behaved in a very similar manner to unirradiated cytochrome c. These results suggested that the 608 mμ chromophore was caused only by alteration of the hematin group and that it still remained attached to the protein. Further attempts were made to fractionate the irradiated cytochrome c on an Amberlite IRC-50 resin by the method of Paleus and Nielands *(11)* but there was no separation of components, again suggesting that the unreacted cytochrome c and the cytochrome c containing the 608 mμ chromophore had very similar properties. Irradiated cytochrome c and pure cytochrome c were treated by the method of Paul *(12)* to split off the hematin group. The spectra of the hematin group from pure cytochrome showed good agreement with that obtained by Paul *(13)*. Comparison of the visible spectra of these two hematins showed that the hematin from irradiated cytochrome c had a stronger absorption band at 580 mμ. Collectively, these properties of irradiated cytochrome c indicate that the 608 mμ chromophore is a result of the oxidation of the porphyrin ring at a methylene group to give an oxyporphyrin, or in terms of the hemoprotein, it is the conversion of the cytochrome c to a verdohemochrome. The most interesting feature of this oxidation of the porphyrin ring of cytochrome c is the large yield obtained as compared to other hematin compounds susceptible to oxidation via ionizing radiation.

Catalase. Catalase showed 37%, 28%, 0%, retention of activity when irradiated in an oxygen atmosphere at doses of 2×10^6, 5×10^6 and 20×10^6 rep, respectively. Catalase showed 115%, 55%, 6% retention of activity when irradiated in a nitrogen atmosphere at doses of 0.3 and 0.5×10^6 rep; 1, 2 and 3×10^6 rep; and 20×10^6 rep, respectively. These results are in agreement with those of others and show that catalase has similar radiation sensitivity as other enzymes, being inactivated by a dose of 20×10^6 rep.

Hematin. The destruction of hematin (Table 5) was a function of the dose of ionizing radiation. Study of the complete spectra showed some indication of reduction of hematin to heme by irradiation at 0.2×10^6 rep.

TABLE 5

Irradiation destruction of hematin

Dose	Destruction measured as hematin	Destruction measured as heme	
	Soret max.	570 mμ max.	Soret max.
r.e.p. $\times 10^{-6}$	%	%	%
0	0	0	0
0.2	13	3	3
2	39	13	27
20	100	100	100

SUMMARY

A survey was made of the effects of gamma radiation on a number of hematin compounds, including hemoglobin derivatives, hemiglobin derivatives, denatured globin hemichromes and cytochrome c. Further evidence was obtained for the anaerobic conversion of methemoglobin to oxyhemoglobin by ionizing radiation. Conditions for maximum production of a 608 mμ chromophore in irradiated cytochrome c were determined. This 608 mμ chromophore is a result of the oxidation of the porphyrin ring in cytochrome c.

Acknowledgments

The assistance of Herman Groninger and Cho C. Tsen is sincerely appreciated.

LITERATURE CITED

1. Barron, E. S. G. The effect of ionizing radiations on systems of biological importance. *Ann. N .Y. Academy Sci.*, **59**, 574 (1955).
2. Barron, E. S. G., and Johnson, P. Studies of the mechanism of action of ionizing radiations. XV. X-irradiation of oxyhemoglobin and related compounds. *Radiation Res.*, **5**, 290 (1956).
3. Benesch, R. E., Lardy, H. A., and Benesch, R. The sulfhydryl groups of crystalline proteins. I. Some albumens, enzymes, and hemoglobins. *J. Biol. Chem.*, **216**, 663 (1955).
4. Erdman, A. M., and Watts, B. M. Radiation preservation of cured meats. *Food Technol.*, **11**, 349 (1957).
5. Ginger, I. D., Lewis, U. J., and Schweigert, B. S. Changes associated with irradiating meat and meat extracts with gamma rays. *J. Ag. Food Chem.*, **3**, 156 (1955).
6. Groninger, H. S., Tappel, A. L., and Knapp, F. W. Some chemical and organoleptic changes in gamma irradiated meats. *Food Research*, **21**, 555 (1956).
7. Hannan, R. S. *Scientific and technological problems involved in using ionizing radiations for the preservation of food.* Special Report No. 61, Her Majesty's Stationery Office, London (1955).
8. Hill, R., and Keilin, D. The porphyrin of component c of cytochrome and its relationship to other porphyrins. *Proc. Roy. Soc.*, **107**B, 286 (1931).
9. Kirn, J. F., Urbain, W. M., and Czarnecki, H. J. Characteristics of electron-irradiated meats stored at refrigerator temperatures. *Food Technol.*, **10**, 601 (1956).
10. Mee, L. K., and Stein, G. The reduction of cytochrome c by free radicals in irradiated solutions. *Biochem. J.*, **62**, 377 (1956).
11. Paleus, S., and Nielands, J. B. Preparation of cytochrome c with the aid of ion exchange resin. *Acta Chem. Scand.*, **4**, 1024 (1950).
12. Paul, K. G. The splitting with silver salts of the cysteine-porphyrin bonds in cytochrome c. *Acta Chem. Scand.*, **4**, 239 (1950).
13. Paul, K. G. The porphyrin component of cytochrome c and its linkage to the protein. *Acta Chem. Scand.*, **5**, 389 (1951).
14. Schuberth, J. Reduction of some haemo proteins by ultraviolet light. *Acta Chem. Scand.*, **10**, 338 (1956).
15. Tappel, A. L. The red pigment of precooked irradiated meats. *Food Research*, **22**, 408 (1957).
16. Tappel, A. L. Regeneration and stability of oxymyoglobin in some gamma irradiated meats. *Food Research*, **21**, 650 (1956).
17. Theorell, H. Chemical constitution of cytochrome c. *Biochem. Z.*, **298**, 242 (1938).
18. Tsou, C. L. Cytochrome c modified by digestion with proteolytic enzymes. I. Digestion. *Biochem. J.*, **49**, 362 (1951).

EXAMINATION OF LEMON OIL BY GAS-PARTITION CHROMATOGRAPHY[a]

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Although gas-partition chromatographic methods have not as yet achieved the complete separation of all of the components of lemon oil, they can and do provide a useful means of examining lemon oils and determining gross compositional differences. In this study thirty-seven samples of both foreign and domestic commercial lemon oil were examined with the aid of gas-partition chromatography and a partial identification of some of the components of the oil was made. The ratio of peak heights of the various components was then determined. By this simple device gross compositional differences in lemon oil by addition of some terpene hydrocarbons can be detected.

EXPERIMENTAL

The gas-partition chromatographic apparatus and ancillary equipment used have been described by Bernhard *(1)* in detail elsewhere.

All analyses were made employing a glass column 11.5 feet long and 6 mm. i.d. filled with a liquid phase consisting of a polyethylene glycol (Carbowax 600) on a support of diatomaceous earth firebrick (Sil-O-Cel C-22, 60–80 mesh) in the proportion 40 g. of Carbowax 600 to 100 g. of firebrick. The carrier gas was helium; inlet pressure was 30 p.s.i.; outlet pressure was atmospheric; flow rate was 25 ml./min.; column temperature was 177°; and a recording potentiometer was used with a chart speed of 16 inches/hour.

RESULTS AND DISCUSSION

Figure 1 presents a typical record of the gas-partition chromatographic separation of some of the components of a domestic cold-pressed lemon oil. The plot shows 5 distinct peaks; the general appearance of the curve is fairly typical for all of the authentic lemon oils examined. By trapping the effluent vapors from the chromatographic column at the appropriate times, the constituents comprising the five fractions of the domestic cold-pressed lemon oil were obtained in sufficient quantity to subject each fraction to examination by infrared spectroscopy. The infrared spectrum of fraction 1 was found to be nearly identical with that of *alpha*-pinene. The differences consist of additional weak bands present in fraction 1 that are not found in the *alpha*-pinene spectrum. Fraction 2 almost certainly contains both the $R_1R_2C{=}CHR_3$ and the isopropyl groups; it is also probable that there are 2 conjugated double bonds present in some compound or compounds present in this fraction. The spectrum of fraction 3 is identical with that of limonene. The major components of fraction 4 appear to contain the $R_1R_2C{=}CHR_3$ and the isopropyl groups. Fraction 5 has an infrared spectrum with a relatively high overall absorption above 6 microns which indicated that it is probably a mixture of several compounds. Hydroxyl, 2 types of carbonyl, $R_1R_2C{=}CHR_3$

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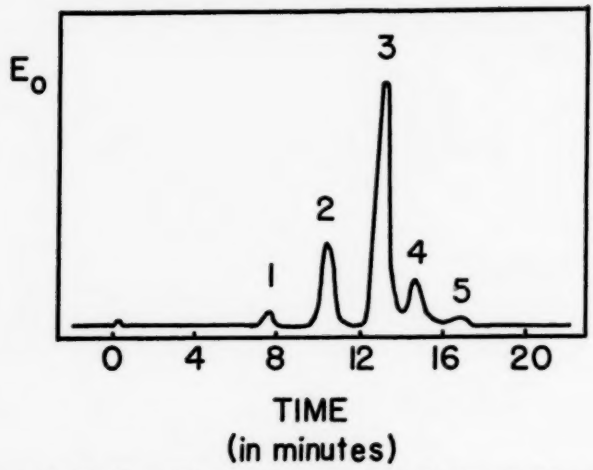

Figure 1. Gas-liquid partition chromatographic examination of cold-pressed lemon oil. Temperature: 177°. Helium inlet pressure, 30 p.s.i.; outlet pressure, atmospheric. Flow rate, 25 ml./min. Liquid phase, Carbowax 600. Chart speed, 16 inches/hour.

and isopropyl groups are very likely present in this fraction. There is also the suggestion that $R_1R_2C{=}CH_2$ may be present.

Twelve samples of authentic domestic cold-pressed lemon oil were examined by means of gas-partition chromatography and the ratio of the peak heights of each of the 5 peaks determined. The variation in peak height ratios among the various oil samples is about twofold. Figure 2 presents a bar graph of the

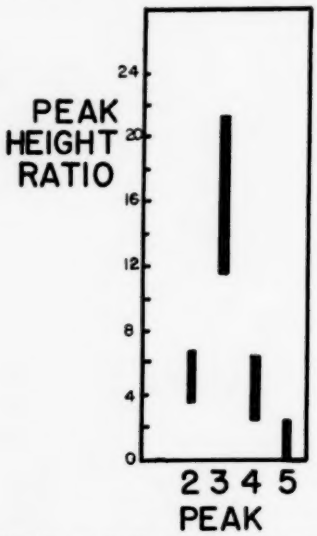

Figure 2. Distribution of peak height ratios for twelve authentic samples of domestic lemon oil.

various peak height ratios compared to peak 1 which has been adjusted to a value of one. Nine samples of authentic foreign lemon oil were examined and the peak height ratios determined as above. In these samples variations were of the same order of magnitude. Figure 3 presents a bar graph of the various

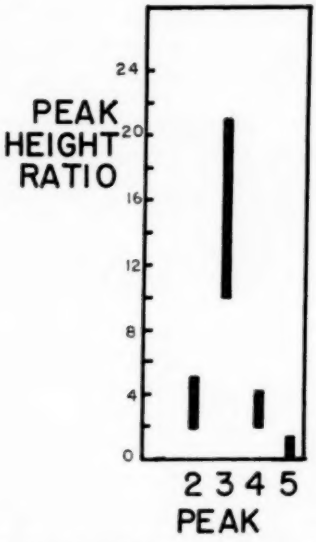

Figure 3. Distribution of peak height ratios for nine authentic samples of foreign lemon oil.

peak height ratios of the oils compared to peak 1 which has been adjusted to a value of one. It should be noted that in some samples of lemon oil, peak 5 was absent. This accounts for the extension of the bars for peak 5 to the base line (see Figures 2 and 3).

The foreign samples tend to show lower peak height ratios which would indicate a higher *alpha*-pinene content in these samples as compared to domestic lemon oils.

Eight domestic and 8 foreign lemon oils of unknown quality were next examined chromatographically and the peak height ratios compared with the respective range of values for the authentic oils. On the basis of the above test, 7 foreign and 4 domestic oils showed unusual peak height characteristics. One imported sample showed an additional peak between peaks 1 and 2 which indicated that a foreign material was present in the oil. The other suspect samples showed very high ratios of peak 3 to peak 1. This evidence seems to support the conclusion that there may be some dilution of the oils by the addition of limonene.

After the above examination was concluded, all 37 oil samples were subjected to the chromatostrip test of Stanley and Vannier *(2)*. This test indicated that of the 37 samples examined, 5 foreign and 4 domestic oils showed unusual differences from the authentic oils. The 5 foreign samples

were part of the seven exhibiting typical peak height ratios, and the 4 domestic samples were identical to the 4 unusual samples found by the peak height ratio method (see above). Thus, there was a high degree of correlation between two widely differing methods of oil analysis. Actually the gas-partition chromatographic method indicated two additional samples were different, which may indicate an increased sensitivity of this method over the chromatostrip procedure.

More precise quantitative procedures for the peak height ratio method are currently being developed and will be reported in the near future.

SUMMARY

A method for the estimation of gross compositional differences between various lemon oils is presented which employs the technique of gas-liquid partition chromatography and the comparison of the peak height ratios of the five fractions separated by this technique.

A partial identification of some of the components and some structural fragments present in lemon oil has been accomplished.

Acknowledgment

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LITERATURE CITED

1. Bernhard, R. A. Separation and identification of some terpenes by gas partition chromatographic analysis. *J. Assoc. Offic. Agr. Chemists*, **40**, 915 (1957).
2. Stanley, W. L., and Vannier, S. H. Analysis of coumarin compounds in citrus oils by liquid solid partition. *J. Assoc. Offic. Agr. Chemists*, **40**, 582 (1957).

NOTES AND LETTERS

PSYCHOPHYSICAL BIAS IN TASTE TESTING BY PAIR COMPARISON, WITH SPECIAL REFERENCE TO POSITION AND TEMPERATURE*

Sensory pair comparison is exposed to psychophysical bias. It is hardly imaginable that a subject's verbal responses to stimuli are not affected by the order of perception; the open questions are the size (it may be negligible) and the control of the effect. Other sources of bias, especially those induced by alien stimuli, are readily envisaged. In the domain of taste, the time-order error has been intermittently studied since its establishment by Fodor and Happisch *(1)* in 1923. Here, it is often synonymous with positional bias; for instance, the left-hand : right-hand alternative ordering of the pair of samples Obviously, samples must be tasted one at a time, and the subject is likely to have an innate locational bias in choosing one of two items. Practical aspects of the phenomenon have been examined by several writers *(3, 4, 5, 7, 8, 9);* this note shows how bias can be built into the conventional probabilistic model for pair comparison, how tasters can be characterized by their biases, and how a temperature bias can affect judgment of taste intensity.

THEORY

Pair comparison of relative taste intensity turns on an unobvious sensory difference. For a particular difference we assume the subject to have a probability P_s of its discrimination. (Perversity, that is, a mistake in the direction of the difference, is not taken into consideration.) The probability of his returning a correct answer to the question, "Which is stronger?", is

$$P = (1 + P_s)/2, \tag{1}$$

and P can be estimated as a fractional frequency, R/N. Let us now define P_b as a probability of bias in the form of a psychophysical tendency to select a particular item in independence from sensory discriminability. The probability of correct selection will then depend upon whether P_b acts with or against P_s. We shall use subscript 1 for "with" and 2 for "against", so that

$$\left.\begin{aligned} P_1 &= P_s + (1 - P_s)(1 + P_b)/2 \\ P_2 &= P_s + (1 - P_s)(1 - P_b)/2 \end{aligned}\right\} \tag{2}$$

If the source of bias is known, the trials can be so split that half estimate P_1 and half P_2. Then

$$P = (P_1 + P_2)/2 = (1 + P_s)/2, \tag{3}$$

which indicates that, as might be expected, the bias cancels in these circumstances. Further, it is easily shown that

$$P_s = P_1 + P_2 - 1, \tag{4}$$

and that

$$P_b = (P_1 - P_2)/(2 - P_1 - P_2), \tag{5}$$

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and that if, in equations (4) and (5), we replace P_1 and P_2 by observed fractional frequencies of correct decisions, the resulting estimates of P_a and P_b will have the property of "maximum likelihood", i.e., they will be the best available ones. It may be noted that the standard errors of these estimates can be approximated from

$$\text{S.E.}(\hat{P}_a) = (P_1Q_1 + P_2Q_2)/N \tag{6}$$

and

$$\text{S.E.}(\hat{P}_b) = \sqrt{\frac{2(P_1Q_1 + P_2Q_2)(Q_1^2 + Q_2^2)/N}{N(Q_1 + Q_2)^2}}, \tag{7}$$

where $Q = 1 - P$, and when the P's are replaced by the fractional frequencies. However, errors as such are less likely to be required than estimates of statistical significance, and these can be obtained by means of X^2-tests with contingency tables—see any standard statistical text.

Bias can be composite. We may replace P_b by, say, P_d, the probability of a directional (positional) bias, and P_t, the probability of attributing a temperature difference to a taste-stimulus difference. [The influence of temperature on taste acuity has been studied by Komura *(6)*, and Goudriaan *(2)*.] Let us use subscript 1 for a trial in which the stronger stimulus is in the warmer solution and on the right-hand side, subscript 2, correspondingly, for stronger-warmer-left, 3 for stronger-cooler-right, and 4 for stronger-cooler-left. Then if N such tetrads of pair comparison are carried out, and if R is the number of correct decisions in each group of N, we have

$$P_a = (R_1 + R_2 + R_3 + R_4)/2N - 1, \tag{8}$$

$$P_t = \frac{(R_1 + R_2) - (R_3 + R_4)}{4N - (R_1 + R_2 + R_3 + R_4)}, \tag{9}$$

$$P_d = \frac{(R_1 + R_3) - (R_2 + R_4)}{4N - (R_1 + R_2 + R_3 + R_4)} \tag{10}$$

as the estimates of the probabilities.

EXPERIMENTAL

An experiment was designed to appraise two components of bias, position and temperature, in simple taste comparisons. A basic mixture of 1 part quinine sulfate, 170 parts tartaric acid, 500 parts sodium chloride, and 3000 parts sucrose, was made up at a 5% level in water. A 15% concentration difference at this level was found to provide a suitable contrast for P_a. Three serving temperatures were chosen for the contrasts: 7° ("cold"), 23° ("room"), and 37° ("hot")—all Celsius. The question put was, "Which member of the pair of solutions (left- or right-hand) has the stronger taste?" and a definite answer was made obligatory. Four subjects participated. Tasting was spread over 12 working days, at 3 daily sessions of 12 randomized and coded pairs per subject. The resultant estimates of the probabilities are assembled in Table 1.

DISCUSSION

Inspection of the Table suggests that, overall, three of the four subjects had a marked right-hand bias, and that the fourth (No. 1) had a slight left-hand bias. Contingency tests show that two of the subjects (Nos. I and III)

TABLE 1

Estimates of probabilities of sensory discrimination and of bias in pair comparison of taste intensity

P_s : sensory discrimination
P_d : tendency to choose the right-hand item (or, if negative, the left-hand)
P_t : tendency to associate the warmer item with a greater (or, if negative, with a lesser) intensity of the taste stimulus

Subject	P_s	P_d				P_t	
	All conditions	No temp. contrast	Room *vs.* hot	Cold *vs.* room	All conditions	Room *vs.* hot	Cold *vs.* room
I	0.63	0	−0.05	−0.32	−0.19	−0.43	0.59
II	0.50	0.69	0.67	0.70	0.69	−0.17	0.52
III	0.42	0.76	0.42	0.28	0.60	0.33	0.74
IV	0.54	0.55	0.31	0.32	0.39	−0.31	0.51
No. of pairs per subject	432	144	96	192	432	96	192

had variable biases over the three test conditions. The temperature bias is evidently not directly related to the temperature of tasting; there is a tendency for both hot and cold—but particularly the latter—to be associated with decrease of stimulus. In other words both heat and cold reduce sensitivity. This interesting observation is reinforced by some auxiliary tests done on pairs that, unknown to the (same four) subjects, had identical concentrations of the taste stimulus. Three temperature contrasts were employed and the pooled results ($N = 4 \times 12$) were

	P_t
cold : room	0.60
cold : hot	0.52
room : hot	−0.18

From Table 1 we also see that those subjects with the highest taste acuity tend to least bias.

As regards positional bias, the moral of this study is always to balance the two (or more) possible presentation arrangements, and to randomize within that restriction; then the bias will cancel. This applies not only to intensity tasting but also, by implication, to qualitative and preference tasting.

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LITERATURE CITED

1. FODOR, K., AND HAPPISCH, L. Über die Verschiedenheit der Unterschiedsschwellen für den Geschmacksinn bei Reizzunahme und Reizabnahme. *Pflüger's Arch.*, **197**, 337–347 (1923).
2. GOUDRIAAN, J. C. Über den Einfluss der Temperature auf die Geschmacksempfindung. *Arch. Neérland. Physiol.*, **15**, 253–282 (1930).
3. HARRIES, J. M. Positional bias in sensory assessments. *Food Technol.*, **10**, 86–90 (1956).

4. HARRISON, S., AND ELDER, L. W. Some applications of statistics to food tasting. *Food Technol.*, **4**, 434–439 (1950).
5. ISHLER, N. H., LAUE, E. A., AND JANNISCH, A. J. Code bias in consumer testing. *Food Technol.*, **9**, 389–391 (1954).
6. KOMURA, K. Le sens du goût a-t-il un coefficient de température? *Arch. Neérland. Physiol.*, **5**, 572–579 (1921).
7. MITCHELL, J. W. The effect of assignment of testing materials to the paired and odd position in the duo-trio taste difference test. *Food Technol.*, **10**, 169–171 (1956).
8. MITCHELL, J. W. Time errors in the paired comparison taste preference test. *Food Technol.*, **10**, 218–220 (1956).
9. SCHWARTZ, N., AND PRATT, C. H. Simultaneous vs. successive presentation in a paired comparison situation. *Food Research*, **21**, 103–108 (1956).

THE ODOR AND FLAVOR OF METHIONAL[a]

Evidence that methional (3-methylthiopropanal) contributes to the sunlight flavor of milk and that it may be broadly significant in the flavor of foods has been published from this laboratory *(2, 3)*. More recently Keeney and Day *(1)* have suggested that methional is important in cheese flavor. On the other hand Witting and Batzer *(6)* have stated that methional, as prepared by them, is *odorless*. We have observed the compound to be intensely odorous and to exhibit an average flavor threshold in skimmilk of 50 parts per billion. These completely divergent findings have necessitated further study of this matter.

Witting and Batzer *(6)* state that their crude reaction mixture containing methional was odorous. However, during purification of the crude product by vacuum distillation the odor seems to have been lost, since distillation "invariably resulted in *odorless* materials". Witting and Batzer state further that such distillates on standing, quickly (3–5 minutes) took on odor which became increasingly intense with time. They presume this change to have resulted from air oxidation.

In an effort to retrace these steps, fresh methional was prepared by the method of Pierson *et al. (4)*. Methyl mercaptan (25 g.) was conducted slowly, during two hours, into 29.2 g. of acrolein under constant agitation and at a temperature of 35–40° C. A drop of pyridine was employed as a catalyst. The crude reaction product was purified in an Ace miniature distillation apparatus. The fraction (42.0 g.) boiling 92 to 95° C. at 59 mm. pressure and with n_D^{20} 1.4790 was taken as the methional. This material had an intense odor, as judged by 6 observers, at the time distillation was complete and the vacuum released. Two redistillations of the methional were made (below 10 mm. pressure) and intermittently during distillation samples of distillate were taken and evaluated for odor by 4 to 6 observers. All such samples and all total distillates were judged to be overwhelmingly odorous in the manner that is characteristic, at least in the opinion of the writers, of methional.

By observing the refractive indices and infrared spectra of freshly distilled samples and aerated samples of methional, evidence of change by air oxidation was sought. Exposure to or even shaking in air for 30 minutes at room temperature had no effect on the refractive index, infrared spectrum or odor quality of any of the methional samples. The stability of the infrared spectrum and refractive index are not necessarily opposed to formation of an extremely potent odor compound in trace amounts. However, no change in odor intensity was noted. In any event the data do not sustain the idea of a rapid, progressive and substantial transformation of freshly distilled material. Thus, it does not appear to us, as suggested by Witting and Batzer, that critical changes presumably involving air oxidation, occur within minutes after distillation to impart odor to methional. Figure 1 presents the infrared spectrum of methional. Rescanning showed no changes. The absorption at 1045 to 1060 cm.$^{-1}$ could conceivably arise from a sulfoxide linkage. However, in

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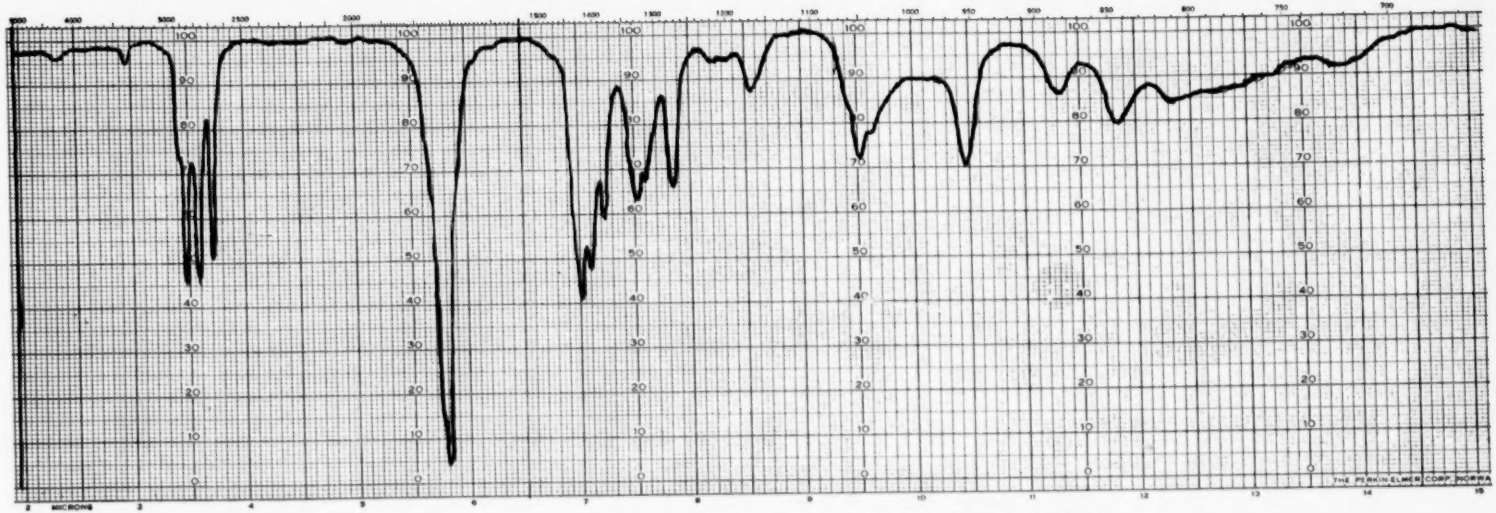

Figure 1. Infrared spectrum of freshly distilled methional (5% by wt. in CCl_4).

light of observations noted below, it seems more plausibly interpreted as one of the characteristic absorption bands for aliphatic aldehydes.

Witting and Batzer have proposed that methional sulfoxide, rather than methional, is the odorous compound in question. To investigate this possibility, 5 mg. each of methionine and methionine sulfoxide were submitted to the Strecker degradation *(5)* with 10 mg. of ninhydrin in 10 ml. of water. This reaction produces the aldehyde of one less carbon from the amino acid and in the case of methionine and its sulfoxide the products would be methional and its sulfoxide, respectively. The samples were heated on a steam bath and although color development with the two compounds appeared comparable, the methionine sample gave strong typical methional odor almost immediately; whereas only a faint atypical odor was evident in the sulfoxide sample after 15 minutes heating. The methionine and its sulfoxide used in the experiment were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Even on theoretical grounds it does not seem reasonable that methional would be completely odorless. It contains two of the most potent odor producing groups known; namely, thioether and aldehyde. Moreover, its chain length is such as to be nearly optimum for odor when compared with such other aliphatic homologous series as alcohols, aldehydes, and acids.

In addition to the various other points discussed here, we could find no support for Witting and Batzer's inference that in the series; sulfide, sulfoxide, sulfone, the sulfoxide is most odorous. Samples in our hands of methyl sulfide, its sulfoxide and sulfone do not bear this out, the sulfide being by far most odorous. In the series methionine, its sulfoxide and sulfone, none appears to be significantly more odorous than the others. The use of methional in the synthesis *(4)* of any of them could impart strong odor by contamination.

The paucity of detail given by Witting and Batzer *(6)* regarding method of synthesis, yield, and properties for their material makes it difficult to account for the divergence in findings. The distillation process appears to be the critical consideration. Further clarification should result from infrared or mass spectral analysis of change in the Witting and Batzer product as it moves from the fresh, odorless to the aged, odorous state.

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LITERATURE CITED

1. KEENEY, M., AND DAY, E. A. Probable role of the Strecker degradation of amino acids in development of cheese flavor. *J. Dairy Sci.*, **40**, 874 (1957).
2. PATTON, S. The mechanism of sunlight flavor formation in milk with special reference to methionine and riboflavin. *J. Dairy Sci.*, **37**, 446 (1954).
3. PATTON, S. The broth-like flavor of methional. *Food Technol.*, **10**, 60 (1956).
4. PIERSON, E., GIELLA, M., AND TISHLER, M. Synthesis of DL-methionine. *J. Am. Chem. Soc.*, **70**, 1450 (1948).
5. SCHÖNBERG, A., AND MAUBASHER, R. The Strecker degradation of α-amino acids. *Chem. Rev.*, **50**, 261 (1952).
6. WITTING, L. A., AND BATZER, O. F. Relation of methional to the odor of irradiated meat. *Food Research*, **22**, 237 (1957).

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